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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

HELDIN et al.

Serial No.: 08/453,350

Group Art Unit: 1646

Filing Date: May 30, 1995

Examiner: C. Saoud

Title: RECOMBINANT PDGF A-CHAIN HOMODIMERS AND METHODS OF USE (AS AMENDED)

Declaration of Lawrence Scott Cousens, P.h.D.

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Lawrence Scott Cousens, declare as follows:

1. I am employed by Chiron Corporation, an assignee of the above-identified application, where I have held the position of Associate Director of Protein Chemistry since 1994. I hold a Ph.D. in Biochemical Sciences from Princeton University and am extremely familiar with protein purification techniques, having actively studied and worked in this discipline for over 25 years. I have coauthored numerous publications in the field of protein chemistry, including publications relating to the purification of a variety of proteins. A true and correct copy of my *curriculum vitae* is attached hereto as Exhibit A.

2. I have reviewed the Office Action dated May 18, 1998 ("the Action"), as well as Heldin et al., *Nature* (1986) 319:511-514 ("Heldin"), cited in the Action. I have also reviewed

the Declaration of Christer Betsholtz, Ph.D., previously submitted. I understand the Office Action disputes the statement made in paragraph 5 of Dr. Betsholtz's Declaration that "methods of purifying proteins from human sources...cannot result in a protein product free of contaminating human proteins." In particular, the Action argues that Heldin's preparation is purified to homogeneity because: (1) "Heldin et al. demonstrate a single band on a silver-stained gel, which is indicative in the art of a homogeneous preparation of protein" (Office Action, page 4, lines 1-2); (2) "Heldin et al. specifically state that 'one homogeneous component of M_r 31,000 was obtained in non-reducing conditions'... The "Declaration has identified no human virus which would copurify with a molecular weight of 31,000" (Office Action, page 4, lines 3-5); and (3) "Heldin et al. state that no other amino acid sequence was obtained from the purified PDGF AA preparation" (Office Action, page 4, lines 5-6) . However, none of the criteria above can be used to support the conclusion that Heldin's preparation was free from human proteins other than the ODGF protein. This conclusion is based on my experience in the field of protein purification and on the facts presented below.

3. In particular, Dr. Betsholtz's previous statement that "methods of purifying proteins from human sources...cannot result in a protein product free of contaminating human proteins" is indeed accurate. It is well known in the field of protein chemistry that no purification technique that uses a human cell as the source of the protein, can render a preparation which is absolutely homogeneous and lacking in other human proteins. This is because no protein purification technique is capable of providing a completely pure product. As stated in the accompanying article by Nobel Laureate Arthur Kornberg, ("Why Purify Enzymes?" in *Methods of Enzymology*, Vol. 182, pp 1-5 (Ed. Murray P. Deutscher), attached as Exhibit B):

> No enzyme is purified to the point of absolute homogeneity. Even when other proteins constitute less than 1% of the purified protein and escape detection by our best methods, there are likely to be many millions of foreign molecules in a reaction mixture. (p. 2, last paragraph, emphasis added.)

Accordingly, a protein purified from human starting material must, by necessity, include other human contaminants, even if these contaminants are undetectable in a silver-stained gel.

4. Furthermore, the limits of detection or sensitivity of silver-stained gels varies greatly from protein to protein. In fact, it is well known that a number of proteins which stain well with Coomassie blue, do not stain at all with silver stains! See, Morrissey, J.H., *Anal. Biochem.* (1981) 117:307-310 ("Morrissey"), attached as Exhibit C. Morrissey explains that silver staining suffers from variable and unreliable staining patterns and has made modifications in an attempt to obtain enhanced uniform sensitivity. However, despite these modifications, it is well known that variation still exists. Further, some low molecular weight proteins may diffuse out of the gel and not be stained at all, some may stain very weakly, and others may stain disproportionately strongly. Moreover, the staining patterns for a given protein may vary considerably from gel to gel. Thus, a single band on a silver-stained SDS polyacrylamide gel, as used in Heldin, does not prove that the product is homogeneous. Had the gel shown in Figure 2 of Heldin been exposed longer, different stains or staining conditions been used, or other methods of protein detection been utilized, human protein contaminants would most certainly have been seen.

5. Additionally, viral contaminants may be present but undetectable using silver staining. Viral contaminants in protein products obtained from human sources are often difficult to detect and do not generally show up with silver staining. In fact, the FDA appears to consider transmittive electron microscopy (TEM) the most sensitive measure of viral contamination. See,

page 23 of "*Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use*," published by the U.S. Department of Health and Human Services, Food and Drug Administration, Center for Biologics Evaluation and Research, February 28, 1997, attached as Exhibit D. Even when TEM is used to assess a protein preparation, such as a monoclonal antibody preparation, the FDA assumes that the titer of virus present is equivalent to 1,000,000/ml. Therefore, it is entirely possible that viral contaminants are present in Heldin's preparation, including contaminants that do not "copurify with a molecular weight of 31,000."

6. Finally, the fact that no other amino acid sequence was obtained from the Heldin preparation does not mean that other human protein contaminants were absent. For example, N-terminal sequencing will not detect contaminants with "blocked" amino termini and many eukaryotic proteins, including some human proteins, are blocked. See, e.g., Driessen et al., *CRC Crit. Rev. Biochem.* (1985) 18:281-325, attached as Exhibit E.

7. The recombinant methods of producing human PDGF A-chain described in the patent application, on the other hand, result in a preparation free of human protein contaminants and devoid of contaminating human viruses. As explained in Dr. Betsholtz's previous Declaration, this is because the only human structural gene present in the recombinant plasmids is the gene encoding human PDGF. It would not be possible to produce preparations having such purity without the gene encoding PDGF and Heldin does not describe the gene or recombinant methods for producing PDGF A-chain.

8. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

10 June 1999
Date

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Publications and Patents:

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References: Supplied upon request.

EXHIBIT B

Methods in Enzymology

Volume 182

Guide to Protein Purification

EDITED BY

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[1] Why Purify Enzymes?

By ARTHUR KORNBERG

"Don't waste clean thinking on dirty enzymes" is an admonition of Efraim Racker's which is at the core of enzymology and good chemical practice. It says simply that detailed studies of how an enzyme catalyzes the conversion of one substance to another is generally a waste of time until the enzyme has been purified away from the other enzymes and substances that make up a crude cell extract. The mixture of thousands of different enzymes released from a disrupted liver, yeast, or bacterial cell likely contains several that direct other rearrangements of the starting material and the product of the particular enzyme's action. Only when we have purified the enzyme to the point that no other enzymes can be detected can we feel assured that a single type of enzyme molecule directs the conversion of substance A to substance B, and does nothing more. Only then can we learn how the enzyme does its work.

The rewards for the labor of purifying an enzyme were laid out in a series of inspirational papers by Otto Warburg in the 1930s. From his laboratory in Berlin-Dahlem came the discipline and many of the methods of purifying enzymes and with those the clarification of key reactions and vitamin functions in respiration and the fermentation of glucose. Warburg's contributions strengthened the *classic approach* to enzymology inaugurated with Eduard Büchner's accidental discovery, at the turn of this century, of cell-free conversion of sucrose to ethanol. One tracks the molecular basis of cellular function—alcoholic fermentation in yeast, glycolysis in muscle, luminescence in a fly, or the replication of DNA—by first observing the phenomenon in a cell-free system. Then one isolates the responsible enzyme (or enzymes) by fractionation of the cell extract and purifies it to homogeneity. Then one hopes to learn enough about the structure of the enzyme to explain how it performs its catalytic functions, responds to regulatory signals, and is associated with other enzymes and structures in the cell.

By a reverse approach, call it *neoclassical,* especially popular in recent decades, one first obtains a structure and then looks for its function. The protein is preferably small and stable, and has been amplified by cloning or is commercially available. By intensive study of the protein and homologous proteins, one hopes to get some clues to how it functions. As the popularity of the neoclassical approach has increased, so has there

been a corresponding decrease in interest in the classical route: pursuit of a function to isolate the responsible structure.

Implicit in the devotion to purifying enzymes is the faith of a dedicated biochemist of being able to reconstitute in a test tube anything a cell can do. In fact, the biochemist with the advantage of manipulating the medium: pH, ionic strength, etc., by creating high concentrations of reactants, by trapping products and so on, should have an easier time of it. Another article of faith is that everything that goes on in a cell is catalyzed by an enzyme. Chemists sometimes find this conviction difficult to swallow.

On a recent occasion I was told by a mature and well-known physical chemist that what fascinated him most in my work was that DNA replication was catalyzed by enzymes! This reminded me of a seminar I gave to the Washington University chemistry department when I arrived in St. Louis in 1953. I was describing the enzymes that make and degrade orotic acid, and began to realize that my audience was rapidly slipping away. Perhaps they had been expecting to hear about an organic synthesis of erotic acid. In a last-ditch attempt to retrieve their attention, I said loudly that every chemical event in the cell depends on the action of an enzyme. At that point, the late Joseph Kennedy, the brilliant young chairman, awoke: "Do you mean to tell us that something as simple as the hydration of carbon dioxide (to form bicarbonate) needs an enzyme?" The Lord had delivered him into my hands. "Yes, Joe, cells have an enzyme, called carbonic anhydrase. It enhances the rate of that reaction more than a million fold."

Enzymes are awesome machines with a suitable level of complexity. One may feel ill at ease grappling with the operations of a cell, let alone those of a multicellular creature, or feel inadequate in probing the fine chemistry of small molecules. Becoming familiar with the personality of an enzyme performing in a major synthetic pathway can be just right. To gain this intimacy, the enzyme must first be purified to near homogeneity. For the separation of a protein species present as one-tenth or one-hundredth of 1% of the many thousands of other kinds in the cellular community, we need to devise and be guided by a quick and reliable assay of its catalytic activity.

No enzyme is purified to the point of absolute homogeneity. Even when other proteins constitute less than 1% of the purified protein and escape detection by our best methods, there are likely to be many millions of foreign molecules in a reaction mixture. Generally, such contaminants do not matter unless they are preponderantly of one kind and are highly active on one of the components being studied.

Only after the properties of the pure enzyme are known is it profitable to examine its behavior in a crude state. "Don't waste clean thinking on dirty enzymes" is sound dogma. I cannot recall a single instance in which I begrudged the time spent on the purification of an enzyme, whether it led to the clarification of a reaction pathway, to discovering new enzymes, to acquiring a unique analytical reagent, or led merely to greater expertise with purification procedures. So, purify, purify, purify.

Purifying an enzyme is rewarding all the way, from first starting to free it from the mob of proteins in a broken cell to having it finally in splendid isolation. It matters that, upon removing the enzyme from its snug cellular niche, one cares about many inclemencies: high dilution in unfriendly solvents, contact with glass surfaces and harsh temperatures, and exposure to metals, oxygen, and untold other perils. Failures are often attributed to the fragility of the enzyme and its ready denaturability, whereas the blame should rest on the scientist for being more easily denatured. Like a parent concerned for a child's whereabouts and safety, one cannot leave the laboratory at night without knowing how much of the enzyme has been recovered in that day's procedure and how much of the contaminating proteins still remain.

To attain the goal of a pure protein, the cardinal rule is that the ratio of enzyme activity to the total protein is increased to the limit. Units of activity and amounts of protein must be strictly accounted for in each manipulation and at every stage. In this vein, the notebook record of an enzyme purification should withstand the scrutiny of an auditor or bank examiner. Not that one should ever regard the enterprise as a business or banking operation. Rather, it often may seem like the ascent of an uncharted mountain: the logistics like those of supplying successively higher base camps. Protein fatalities and confusing contaminants may resemble the adventure of unexpected storms and hardships. Gratifying views along the way feed the anticipation of what will be seen from the top. The ultimate reward of a pure enzyme is tantamount to the unobstructed and commanding view from the summit. Beyond the grand vista and thrill of being there first, there is no need for descent, but rather the prospect of even more inviting mountains, each with the promise of even grander views.

With the purified enzyme, we learn about its catalytic activities and its responsiveness to regulatory molecules that raise or lower activity. Beyond the catalytic and regulatory aspects, enzymes have a social face that dictates crucial interactions with other enzymes, nucleic acids, and membrane surfaces. To gain a perspective on the enzyme's contributions to the cellular economy, we must also identify the factors that induce or

repress the genes responsible for producing the enzyme. Tracking a metabolic or biosynthetic enzyme uncovers marvelous intricacies by which a bacterial cell gears enzyme production precisely to its fluctuating needs.

Popular interest now centers on understanding the growth and development of flies and worms, their cells and tissues. Many laboratories focus on the aberrations of cancer and hope that their studies will furnish insights into the normal patterns. Enormous efforts are also devoted to AIDS, both to the virus and its destructive action on the immune system. In these various studies, the effects of manipulating the cell's genome and the actions of viruses and agents are almost always monitored with intact cells and organisms. Rarely are attempts made to examine a stage in an overall process in a cell-free system. This reliance in current biological research on intact cells and organisms to fathom their chemistry is a modern version of the vitalism that befell Pasteur and that has permeated the attitudes of generations of biologists before and since.

It baffles me that the utterly simple and proven enzymologic approach to solving basic problems in metabolism is so commonly ignored. The precept that discrete substances and their interactions must be understood before more complex phenomena can be explained is rooted in the history of biochemistry and should by now be utterly commensensical. Robert Koch, in identifying the causative agent of an infectious disease, taught us a century ago that we must first isolate the responsible microbe from all others. Organic chemists have known even longer that we must purify and crystallize a substance to prove its identity. More recently in history, the vitamin hunters found it futile to try to discover the metabolic and nutritional roles of vitamins without having isolated each in pure form. And so with enzymes it is only by purifying enzymes that we can clearly identify each of the molecular machines responsible for a discrete

FIG. 1. Personalized license plate expressing a commitment to enzymology.

metabolic operation. Convinced of this, one of my graduate students expressed it in a personalized license plate (Fig. 1).

Acknowledgment

This article borrows extensively from "For the Love of Enzymes: The Odyssey of a Biochemist," Harvard University Press, 1989.

EXHIBIT C

ANALYTICAL BIOCHEMISTRY 117, 307–310 (1981)

Silver Stain for Proteins in Polyacrylamide Gels: A Modified Procedure with Enhanced Uniform Sensitivity

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Received May 4, 1981

The rapid, ultrasensitive silver stains that have been developed recently for detecting proteins in polyacrylamide gels show variation in staining from gel to gel and do not stain certain proteins at all. It was found that treatment of gels with dithiothreitol prior to impregnation with silver nitrate results in more reproducible staining patterns that are also qualitatively similar to those obtained with Coomassie blue. In addition, it obviates the need for treatment with intense light, and results in sensitivities at least as high as those obtained with previously published methods.

The original silver stain for detecting polypeptides in polyacrylamide gels (1) has been simplified and reduced in cost by several groups (2–4). These staining procedures are some 100-fold more sensitive than Coomassie blue, making them extremely useful for detecting proteins in trace quantities and for staining two-dimensional gels (3,4). I have found that there is considerable variation from gel to gel in sensitivity and that some proteins which stain well with Coomassie do not stain at all with silver stains. Other drawbacks to these procedures are that some require special high-intensity light sources (3,4), employ unstable, potentially explosive solutions (1,2), or result in surface staining of the gels, which causes gels to stick to both the container and to each other (1,2). This latter point means that gels must be stained in separate containers. Since development of these stains is stopped at an arbitrary point, different gels will be stained to different extents, making direct comparisons between gels difficult.

This report describes a silver-staining procedure that is more constant from gel to gel, uses stable solutions, is independent of lighting conditions, stains certain proteins not stained by other silver stains, and can be used to stain a number of gels in one container.

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MATERIALS AND METHODS

Electrophoresis. Sample preparations and electrophoresis were performed according to Laemmli (5) in slab gels containing 10% acrylamide. Bromphenol blue was the tracking dye.

Proteins. Dictyostelium discoideum (strain NC4) spores were lysed in a French pressure cell and the lysate was then boiled for 10 min in sample buffer. *D. discoideum* spore coat proteins were prepared according to Orlowski and Loomis (6). Molecular-weight standards were obtained from the following sources: rabbit muscle myosin, β-galactosidase, conalbumin, cytochrome *c*, and insulin from Sigma; ovalbumin from Miles. Discoidin was a gift from Dr. B. Murray.

Coomassie blue stain. Gels were soaked for several hours in 0.2% Coomassie blue R-250, 45% methanol, 10% acetic acid and then destained in 10% methanol, 7% acetic acid.

Silver stains. Gels were stained by the method of Oakley *et al.* (2) as published or

by the method of Merril *et al.* (4) using a No. 1 photoflood lamp equipped with an aluminum reflector illuminating the gel at a distance of 10 cm.

The new silver stain is performed as follows (gentle but thorough agitation is important throughout the procedure):

Step 1: Prefix the gel in 50% methanol, 10% acetic acid for 30 min, followed by 5% methanol, 7% acetic acid for 30 min.

Step 2: Fix the gel for 30 min in 10% glutaraldehyde (E. M. Sciences, biological grade).

Step 3: Rinse the gel in distilled water. It is most convenient to soak the gel in a large volume of water overnight, followed by a fresh water rinse the next day for 30 min. Alternatively, wash in running deionized water, or several changes of water, for 2 h.

Step 4: Soak in 5 μg/ml dithiothreitol for 30 min.

Step 5: Pour off solution and without rinsing, add 0.1% silver nitrate. Treat for 30 min.

Step 6: Rinse once rapidly with a small amount of distilled water and then twice rapidly with a small amount of developer. Soak in developer (50 μl of 37% formaldehyde in 100 ml 3% sodium carbonate) until the desired level of staining is attained. Staining is stopped by adding 5 ml of 2.3 M citric acid directly to the developer and agitating for 10 min. This solution is then discarded and the gel is washed several times in distilled water over a 30-min period. For storage, it is best to soak the gel for 10 min in 0.03% sodium carbonate (to prevent bleaching), and then either to seal in heat-sealable food storage bags or wrap in cellophane.

Gels are fixed and stained in glass or polyethylene containers; since silver is not deposited on surfaces, no special cleaning is required. The same container may be used throughout the procedure. For gels of 1-mm × 9-cm × 13-cm dimensions, all volumes are 100 ml per gel, except for the 10% glutaraldehyde, which is 50 ml. These volumes should be adjusted accordingly if different size gels are used. Particular attention should be paid to the volumes of the carbonate and citric acid solutions, which must be balanced to bring the pH to neutrality. If the pH remains too high, the reaction will not stop, and if the pH falls too low, the gel will bleach.

Gels should be handled only with rinsed plastic gloves to avoid stained fingerprints. Another source of contamination that can cause spurious staining is dust in the gel solutions (Fig. 2). All solutions must be filtered through Millipore filters.

Gels were photographed on Polaroid type 55 film using a Wratten No. 45 filter for silver-stained gels or a No. 15 filter for Coomassie-stained gels.

RESULTS AND DISCUSSION

In the course of using published silver stains for gels, I have found that certain proteins repeatedly fail to show any staining, while the staining of other proteins varies considerably from gel to gel. As may be seen in Fig. 1, the *D. discoideum* spore coat protein sp96 (6), identified in lanes 2 at the position marked with triangles, stains with Coomassie blue (panel A), but not with the silver-staining methods of Merril *et al.* (4) (panel B) or Oakley *et al.* (2) (panel C). This protein has failed to stain with either silver stain in at least 20 attempts. When molecular-weight marker proteins are stained, both silver stains show reduced sensitivity to insulin B chain, and in this gel the method of Merril *et al.* (4) (panel B) shows little sensitivity to cytochrome *c*. Occasionally, the method of Oakley *et al.* (2) has been found to give a staining pattern with insulin B chain and bovine serum albumin which consists of a ring of staining surrounding an unstained band. In some cases with this procedure conalbumin exhibits negative staining, in that an unstained area considerably lighter than the background is found in place of a stained band (not shown).

Variable and unreliable staining reactions

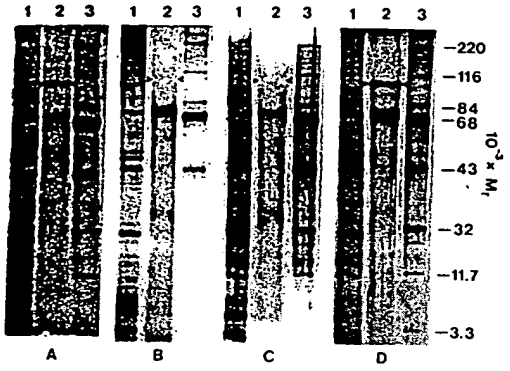

FIG. 1. Comparison of staining techniques. In A, lane 1 contains 50 μg total *D. discoideum* spore proteins, lane 2 contains *D. discoideum* spore coat proteins, (sp96 is identified by triangles), and lane 3 contains 2 μg each of the following proteins (molecular weight given in parentheses): myosin (220,000), β-galactosidase (116,000), conalbumin (84,000), bovine serum albumin (68,000), ovalbumin (43,000), discoidin I (32,000), cytochrome *c* (11,700), and insulin B chain (3300). This gel was stained with Coomassie blue. B–D contain the same samples diluted 1:33. Staining procedures are: B, Ref. (4); C, Ref. (2); D, this report. Proteins were loaded into 6-mm-wide slots.

have been observed with histological silver stains; however, Sun and Green (7) have found that treating tissues with 2-mercaptoethanol immediately prior to silver impregnation gives much more reproducible results. Their reasoning is that since silver staining likely involves reduction of silver ions to silver metal by proteins, full sensitivity will be obtained when the proteins are fully reduced. Variability of staining can thus be explained by postulating varying degrees of oxidation experienced by the tissues during handling. Accordingly, it was found that treating gels with dithiothreitol before silver nitrate impregnation results in staining patterns that are more reproducible for the procedure of Merril *et al.* (4), dithiothreitol treatment replaces the dichromate step). A surprising bonus is that such treatment makes the photochemical method of Merril *et al.* (4) independent of lighting conditions: full sensitivity is obtained in complete darkness. It was also found that other reducing agents (2-mercaptoethanol and chloral hydrate) had similar effects. However, only dithiothreitol was effective at sufficiently low concentrations as to keep background staining to a minimum.

These findings were used to develop a new procedure based in part on previously published methods (2,4) and employing dithiothreitol reduction. As may be seen in Fig. 1D, sp96 stains well with the new silver stain, as do all of the molecular-weight marker proteins. It may also be seen that the total cellular protein pattern qualitatively resembles that of the Coomassie blue-stained lane. This procedure has been found to be substantially more reproducible from gel to gel than the published procedures quoted above.

Among the advantages of this procedure are: (i) As with other rapid silver stains (3,4), it uses only small amounts of silver (as of April 1981 it cost 3.8 cents in silver nitrate to stain one gel). (ii) It does not require special high-intensity light sources. (iii) Unlike ammoniacal silver stains, it does not employ unstable or potentially explosive solutions. (iv) There is virtually no surface

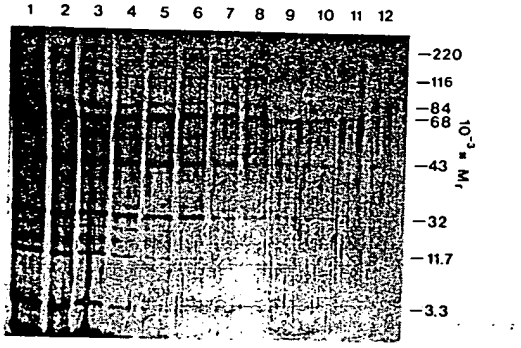

FIG. 2. Sensitivity of the new silver stain. Dilutions of the sample mixture used in lanes 3 in Fig. 1 were applied to this gel. The amount of each protein (in ng) loaded per lane is: lane 1 = 100, 2 = 50, 3 = 25, 4 = 10, 5 = 7.5, 6 = 5, 7 = 3.5, 8 = 2, 9 = 1.5, 10 = 1, 11 = 0.5, 12 = 0.25. The staining seen across the gel just below bovine serum albumin is due to a contaminant in the sample buffer which stains when gels are developed to maximum sensitivity. The thin vertical streaks visible in this gel and in Fig. 1D are from dust particles and can be avoided by filtering all of the gel solutions before use.

deposition of silver, substantially reducing background staining. Since surface staining causes gels to stick together, this improvement means that several gels may be stained together in one container. All of the gels are stained to the same extent, so that quantitative comparisons may be made between gels run in parallel.

As may be seen in Fig. 2, this method is at least as sensitive as the previously published methods. Sensitivities of selected proteins are: 0.042 ng/mm^2 for bovine serum albumin, 0.083 ng/mm^2 for ovalbumin, and 0.17 ng/mm^2 for cytochrome *c*. These values compare favorably to those of Oakley *et al.* (2) and Switzer *et al.* (1). I have found that silver-stained gels may be fluorographed successfully, using the commercially available preparation Enhance (New England Nuclear): even heavily overstained gels of [^{35}S]methionine-labeled bacterial proteins gave fluorographic patterns only slightly reduced in intensity compared to unstained gels (data not shown). However, silver-staining severely quenches tritium fluorography.

ACKNOWLEDGMENTS

I would like to acknowledge the help and advice of Drs. J. Schmidt, K. Devine, and B. Murray and Dr. W. F. Loomis, in whose laboratory these experiments were conducted. This work was supported by a grant to W.F.L. from the National Science Foundation (PCM 79-02698). J.H.M. was supported by an institutional postdoctoral training grant from the National Institutes of Health (GM 07199-06).

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A Simple Procedure
Dilute Solutions

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National Institutes of Health.

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ent address: National Institute of A
Diseases, Bethesda, Maryland 20
[2] To whom all correspondence

EXHIBIT D

Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use

U. S. Department of Health and Human Services
Food and Drug Administration
Center for Biologics Evaluation and Research
February 28,1997

Date: February 27, 1997

From: Kathryn C. Zoon , Ph.D., Director
Center for Biologics Evaluation and Research
Food and Drug Administration

Subject: Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use

To: Manufacturers of Biological Products and Other Interested Persons

This Points to Consider (PTC) document has been developed for manufacturers of monoclonal antibody products for human use. These "Points" are not regulations nor are they guidelines, but represent the current thinking that the Center for Biologics Evaluation and Research (CBER) staff believe should be considered at this time. This 1997 PTC document supersedes the 1994 PTC document of the same title, announced in the Federal Register of August 3, 1994 (59 FR 39571).

It is our intention to continuously update and revise this document in order to improve its usefulness. We invite your review and comment on the "Points". Comments should be identified with the docket number 94D-0259. Two copies of any comments should be submitted except that individuals may submit one copy. All comments should be addressed to:

Dockets Management Branch (HFA-305)
Food and Drug Administration
12420 Parklawn Drive, Room 1-23
Rockville, MD 20857

-s-
Kathryn C. Zoon, Ph.D.

Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use

[Docket No. 94D-0259]

For further information regarding this document, contact:
Sharon Carayiannis
Center for Biologics Evaluation and Research (HFM-630)
Food and Drug Administration
1401 Rockville Pike, Suite 200N
Rockville, MD 20852-1448
301-594-3074

Submit written comments on this document to:

Dockets Management Branch (HFA-305)
Food and Drug Administration
12420 Parklawn Drive, Room 1-23
Rockville, MD 20857

Comments should be identified with the docket number found in the heading of this page.

Submit written requests for additional copies of this document or any other CBER guidance to:

Office of Communication, Training, and Manufacturers Assistance (HFM-40)
Food and Drug Administration
1401 Rockville Pike, Suite 200N
Rockville, MD 20852-1448

Send one self-addressed adhesive label to assist that office in processing your request.

These documents may also be obtained by mail by calling the CBER Voice Information System at 1-800-835-4709 or 301-827-1800, or by fax by calling the FAX Information System at 1-888-CBER-FAX or 301-827-3844.

Persons with access to the INTERNET may obtain these documents using, the World Wide Web (WWW), or bounce-back e-mail. For WWW access, connect to CBER at "http://www.fda.gov/cber/cberftp.html". To receive this document by bounce-back e-mail , send a message to "ptc__mab@A1.CBER.FDA.GOV".

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Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use February 1997

I. INTRODUCTION

A. BACKGROUND

Points to Consider documents provide a flexible approach in which FDA provides and updates its guidance on regulatory issues in many areas of drug development. Such documents are particularly useful in the rapidly evolving field of biotechnology-derived drugs and other biologics. The Center for Biologics Evaluation and Research (CBER) set out to revise the "Points to Consider (PTC) in the Manufacture and Testing of Monoclonal Antibody Products for Human Use" with several objectives. An important goal was to facilitate initial development of monoclonal antibodies for serious or life threatening indications. Additionally, it was felt that some of the information in the 1994 document required updating and streamlining. Finally, it was necessary to review this document for consistency with current CBER policy and with International Conference on Harmonisation (ICH) documents dealing with this category of products. This updated document supersedes the 1994 version, and is designed to assist sponsors and investigators regarding monoclonal antibody (mAb) product development, including information to submit when filing Investigational New Drug Applications ("INDs") and License Applications. Although this document does not create or confer any rights for or on any person and does not operate to bind FDA or the public, it does represent the agency's current thinking on monoclonal antibody products for human use.

For mAb, as for other biologics, certain regulations contained in 21 CFR Parts 200-299 and 600-680 apply and should be consulted. In common with the other PTC, the mAb PTC are not intended to be all-inclusive. They represent recommendations on how to conduct the clinical development of a product up to and after licensure, not checklists of items to be provided before or after phase 1 trials are initiated. Specific products which raise issues that are not considered in these "Points" will be evaluated on a case-by-case basis. The discussion on abbreviated product testing for feasibility trials in serious and immediately life-threatening conditions and on generic and modular virus clearance studies does not apply to human products made in human cell substrates. Consultation with CBER is strongly advised for sponsors considering the application of abbreviated testing policies to products that have the potential to be contaminated by human pathogens. For aspects of manufacturing and of the production facility that are not included in this discussion or in applicable regulations, sponsors should consult with the Office of Therapeutics Research and Review and the Office of Establishment Licensing and Product Surveillance respectively.

B. DEFINITIONS

For the purpose of this document, the terms **"antibody"** and **"monoclonal antibody"** (mAb) may be used interchangeably and refer to intact immunoglobulins produced by hybridomas, immunoconjugates and, as appropriate, immunoglobulin fragments and recombinant proteins derived from immunoglobulins, such as chimeric and humanized immunoglobulins, F(ab') and $F(ab')_2$ fragments, single-chain antibodies, recombinant immunoglobulin variable regions (Fvs) etc. Recommendations on the manufacture of recombinant products are contained in other PTC documents from CBER (1,2). Some of these recommendations pertaining to recombinant mAb produced in cell substrates other than hybridomas are reiterated in this document for convenience of consultation. This document applies to mAb used as therapeutic or *in vivo* diagnostic agents, as well as to **ancillary products**, i.e. mAb used in the manufacture of other products for *in vivo* use. The latter include mAb that are used alone or in

conjunction with devices, for example, for *ex vivo* purging of cells to remove immune or tumor cells, for *ex vivo* cell collection (e.g. hematopoietic stem cells), or for purification of other products intended for *in vivo* administration. Generally, these mAb should meet the same criteria for safety and freedom from adventitious agents as mAb intended for direct administration to patients. Likewise, reagents that are commonly used in conjunction with mAb for *ex vivo* manipulations of cellular products intended for *in vivo* administration (e.g. complement, DNAase) should meet the same safety standards as mAb intended for direct administration to patients. However, in such cases, some procedures for virus inactivation or removal may be performed on the downstream product rather than on the mAb or other reagent (see II.C.7). Complete information on products that will be used in conjunction with the mAb, such as rabbit complement or DNAase, should be submitted before clinical studies begin. This information can be submitted as a part of the original IND submission or in the form of a Master File.

As used in this document, "**cocktails**" are defined as two or more mAb administered at a fixed ratio. Relevant targets may include multiple antigens on infectious pathogens and multiple tumor-associated antigens. The rationale for combining the products should be clear and based on the clinical context or previous clinical experience with individual products. Lack of interference among the mAb in the combination should be shown and synergistic or additive effects should be characterized. Dose-ranging for each of the components is highly desirable. In some instances, dose-setting may be based on preclinical or clinical data that show the necessity or superiority of a particular dose and ratio of mAb in the combination.

As used in this document, "**panels**" are defined as sets of mAb directed against related antigens from which one or more members would be used for an individual patient based on target antigen characterization. Such panels could be submitted for approval in a single license application. Examples of panels might include anti-idiotype mAb for lymphoma and mAb directed against different bacterial or viral serotypes. Dose-ranging for each mAb would be necessary. During the phase 3 trials to establish efficacy of the entire panel, some clinical experience with each member of the panel should be obtained.

C. FILING INFORMATION

It is not necessary to have all of the information discussed in this document available in the initial IND submission. Rather, much of the information may be developed during clinical development, with guidance from CBER or other appropriate Centers by means of frequent dialogue. At pre-IND meetings, CBER staff may provide guidance in planning clinical development and establishing the format and content of initial IND submissions. Such meetings may be particularly useful when the product is a novel molecular entity or is produced by a novel process, and when drug development plans are unusually complex.

The manufacture of mAb that are produced and controlled by similar procedures in the same facility may in some cases be documented in a single Master File. This may be particularly helpful when data from generic or modular virus clearance studies are used for multiple antibodies that differ only in the variable (v) or complementarity-determining region (CDR) and when multiple antibodies are purified by identical procedures (see Section II.C.6).

See references 3 and 4 for information on filing biologics license applications. An Establishment License Application is no longer required for mAb intended for in vivo use (3).

II. PRODUCT MANUFACTURE AND TESTING

A. GENERAL PRINCIPLES AND DEFINITIONS

Traditionally, most mAb are produced by hybridoma cell lines through immortalization of antibody-producing cells by chemically-induced fusion with myeloma cells. In some cases, additional fusions with other lines have created "triomas" and "quadromas". We anticipate an increase in recombinant mAb (e.g. chimeric or humanized mAb, single-chain or dimeric Fvs, mAbs derived from phage display libraries etc.) and human mAb in the future. These may be produced in animal cell lines (e.g. CHO, SP2/0) transfected with recombinant DNA constructs, in human cells (e.g. immortalized lymphoid cells), in bacteria, yeast, insect cells etc. Novel methods of production for mAb or mAb-derived recombinant proteins may include insects, plants or transgenic animals,.
The principles reviewed in Sections II.B. 1 through 4 may be applied, in general to all hybridoma and heterohybridoma generated products, regardless of the species of origin. All steps in manufacturing of mAb to be used in trials intended to support licensure and of licensed mAb should comply with current Good Manufacturing Practices (cGMPs), as appropriate for the stage of product development.

While manufacturing details and safety issues may be different for different expression systems, some general principles can be applied. The establishment of a reliable and continuous source from which the antibody can be consistently produced is highly recommended (e. g. master cell banks for cell cultures, seed banks for transgenic plants, founder strains for transgenic animals). If transient expression systems are used, master vector seed stocks should be generated, and the genetic stability of the expression constructs used should be tested. Appropriate in-process testing which takes into consideration the specific safety concerns of the expression system used should be instituted. Sponsors are encouraged to consult the most recent available versions of the Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals, the Points to Consider in the Production and Testing of New Drugs and Biologicals produced by Recombinant DNA Technology or the Points to Consider in the Manufacture and Testing of Therapeutic Products for Human Use Derived From Transgenic Animals (1, 2, 5), the 1996 CBER/CDER Guidance Document on the Submission of Chemistry, Manufacturing and Controls Information for a Therapeutic Recombinant DNA-derived Product or a Monoclonal Antibody Product for In Vivo Use (4), as well as relevant International Conference on Harmonization (ICH) documents (e.g. 6, 7), if applicable to their expression systems. Sponsors considering novel expression systems not specifically covered by guidance documents are encouraged to consult with CBER.

B. MANUFACTURE AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES

1. Cell lines

The following information should be provided in the IND or biologics license application:

a. Source, name, and characterization of the parent cell line, including any immunoglobulin heavy or light chains that it synthesizes and/or secretes, the fusion partner in the case of hybridomas, or the host cell line in the case of transfected cells producing recombinant mAb.

b. Species, animal strain, characterization, and tissue origin of the immune cell.

c. Description of immortalization procedures, if any, used in generating the cell line.

d. Identification and characterization of the immunogen. A complete biochemical characterization may

not be possible or necessary in all cases. However, we recommend that as much information as possible be gathered on the nature and characteristics of the material used as an immunogen. Such data can be useful in choosing appropriate potency assays, as well as in evaluating potential for cross-reactivity and possible clinical usefulness. For example, a determinant which is not expressed on the surface of target cells bind necrotic cells better than intact cells.

e. Description of the immunization scheme. In the case of human mAb, any *in vitro* or *in vivo* immunization procedures should be described, as well as any relevant aspects of the subject's medical history.

f. Description of the screening procedure used. In the case of human mAb, steps performed in order to enrich antigen-specific human B cell populations should be described.

g. Description of the cell cloning procedures. If changes in cell culture process (e.g. cells adapted from serum-containing to serum-free medium) are shown not to affect product quality, it is not necessary to reclone the cells or rebank the MCB or WCB. In this context, product quality includes not just the identity, purity, potency and pharmacological characteristics of the purified product, but also its safety profile. For example, possible changes in types and/or titers of viruses detectable in the unpurified bulk material and the ability of the purification process to remove or inactivate them should be addressed).

h. For transfected animal or plant cell substrates, as well as for microbial cell substrates (bacteria, yeast), a detailed description of the vector(s) and final construct(s) generation, including whether or not extraneous amino acid sequences are introduced into the product as a result of subcloning, and description of transfection/transformation, screening and selection procedures (see refs. 1-6). Determination of cDNA sequence(s) of the predominant transcript(s) is acceptable as an indication of clonality of transfected cell lines.

i. For cell culture systems using autonomously replicating vectors (e.g. baculovirus or other transient expression systems) a detailed description of the vector system, construct generation, selection, vector banking procedures, and infection/transfection procedures should be provided (1-6).

j. For all cell substrates, description of the seed lot system for establishing and maintaining the master cell bank (MCB) and the working cell bank (WCB).

2. Production in cell culture

The following information should be provided in the IND or biologics license application:

a. A description of the culture procedures if production is entirely *in vitro* or if cells are passaged *in vitro* prior to mouse inoculation.

b. A description of the culture media used, including certification and testing. Serum additives used in hybridoma propagation should be free of contaminants and adventitious agents.

c. The steps taken to prevent or control contamination by viruses, bacteria, fungi, mycoplasma and transmissible spongiform encephalopaties (TSE) agents. These include, among other things, a description of the equipment, transfers, room classification, employee gowning procedures etc.

d. The acceptance criteria for cells or tissue culture supernatants intended for further manufacture.

3. Production in animals or plants

The following information should be provided in the IND or biologics license application:

a. A description of the cell line used as the inoculum (if any) should be provided (see 2.a. above).

b. Animal care should be in accordance with the NIH Guide for Care and Use of Laboratory Animals. For ascites production, the use of specific pathogen free (SPF) mice is recommended. To ensure manufacture of consistent, high quality ascites for production of mAb, an animal health monitoring program should be in place that encompasses quarantine procedures, sentinel animals, and an in-house health surveillance program (including screening for mycoplasma). Frequency of serological testing of sentinel mice should be established and is usually based on the incidence of virus contamination. Screening programs for known infectious agents should be updated to reflect advances in the knowledge of infectious diseases. Sponsors should be responsible for the adequacy of screening programs.

c. All protocols for ascites production should also incorporate information on: *i*) species, sex and age of animals used; *ii*) animal supplier; *iii*) volume of pristane; *iv*) volume and concentration of cell inoculum; *v*) timing of priming, inoculation, and ascites harvesting; *vi*) frequency and procedure for ascites harvesting; *vii*) definition of a batch; *viii*) animal bedding, food and water; *ix*) number of animals housed together; *x*) environmental conditions under which each procedure takes place and *xi*) number of times cells are passaged from one animal to another, if applicable.

d. For production in transgenic animals, the vectors, constructs and procedures used for gene transfer should be described. The genetic background and characterization of founder animals, the generation and selection of production herds and animal maintenance procedures should be described as well (see ref. 5 for details). Health monitoring programs for animal herds or colonies should be in place, including screening for zoonoses known to exist in captive animals of the relevant species in North America. Programs for screening and detection of known infectious agents should be tailored for the animal species and periodically updated to reflect advances in the knowledge of infectious diseases. Sponsors should be responsible for the adequacy of screening programs (see paragraph b above). When initially establishing transgenic animal strains, the following considerations should be kept in mind: *i*) non-transgenic animals to be used for breeding or gene transfer procedures should be obtained from closed herds or colonies that are serologically well characterized and as free as possible of pathogens of concern for the animal species or for humans; *ii*) the use of imported animals or first generation offspring from imported animals is discouraged and *iii*) animals from species in which TSE have been documented should be obtained from closed herds with documented absence of dementing illnesses and controlled food sources.

e. For production using autonomously replicating vectors (e.g. baculovirus) in live insect larvae, larvae maintenance procedures should be described in detail, including procedures used to control and monitor bioburden.

f. For production in plants (e.g. transgenic plants or autonomously replicating vectors using plants as bioreactors), early consultation with CBER staff is recommended.

4. Purification

Purification schemes for mAb should be described in the IND or biologics license application. We recommend that mAb purification schemes incorporate the following characteristics:

a. Production techniques that will prevent the introduction of and eliminate contaminants, including

animal proteins and materials, DNA, endotoxin, other pyrogens, culture media constituents, components that may leach from columns, and viruses.

b. Incorporation of one or more steps known to remove or inactivate retroviruses in excess of the endogenous particle load, whenever applicable (see Reference 8 and discussion of virus clearance studies in Section II.C.). As a general guidance, we recommend that each purification protocol include at least two orthogonal (i.e. based on different mechanisms) robust virus removal steps (see below). Including these steps would not obviate the need for virus clearance studies, except in the case of products intended for use in feasibility trials in serious or life-threatening conditions (see Section II.D.2.)

> *i.* **Robust virus removal/inactivation steps** are defined as those that have been shown to work well under a variety of conditions (e.g. pH or ionic strength of column buffers) with a variety of mAb. Robust steps include low pH, heat, solvent/detergent treatments, and filtration (see Table III). Sponsors have the option of providing adequate evidence indicating that a step different from these is robust, or is reliably effective for removal/inactivation under the conditions employed. An estimate of the efficiency of robust steps in removing virus may be demonstrated by: (a) cross-referencing master files or reliable scientific literature published in peer-reviewed journals or (b) generic or modular clearance studies (see Section II.C.6. for definition).

c. Demonstration of the ability of the purification scheme to remove adventitious agents and other contaminants, by means of a clearance study. For some contaminants, e.g. DNA, pristane or protein A, such a clearance study, if appropriately carried out, may be an acceptable alternative to routine testing for the contaminant. In the case of virus clearance studies, we recommend the use of several model viruses encompassing large and small particles, DNA and RNA genomes, as well as chemically sensitive and resistant lipid enveloped and non-enveloped strains. Human blood products should be avoided in production of other biologicals. When human blood products that may be contaminated with hepatitis C virus (HCV) or other infectious agents must be used in production (e.g. as media additives), such schemes should include viruses that are acceptable models for HCV, such as bovine viral diarrhea virus (BVDV) or Sindbis virus. Retrovirus clearance studies should be performed prior to phase 1 trials, except for products intended for use in the setting of serious or life-threatening conditions in feasibility trials (see Section II.D.). Clearance studies for other viruses and/or other contaminants should be carried out prior to production for phase 2/3 trials and may need to be repeated if the final manufacturing process has changed. ICH guidelines are currently being drafted to address in further detail the viral safety evaluation of biotechnology products derived from cell lines of human or animal origin.

d. Limits should be prospectively set on the number of times a purification component (e.g. a chromatography column) can be reused. Such limits should be based upon actual data obtained by monitoring the component's performance over time.

e. As a product is developed, retention samples from each production should be saved under appropriate conditions so that side-to-side comparisons may be made to determine product comparability (see Section II.E.).

f. A description of the purification room(s) design features, HVAC and other support systems, equipment, transfers and personnel should be provided. Emphasis should be placed on operational features that minimize the risk of contamination from the environment or cross-contamination from other products.

5. Characterization of purified unmodified mAb

Before a mAb is studied in humans, a precise and thorough characterization of antibody structural

integrity, specificity, and potency should be conducted and described in the IND. The mAb should be as free as possible of non-Ig contaminants. A properly qualified in-house reference standard with known characteristics, specificity, and potency, and that is stored under appropriate conditions and periodically tested to ensure its integrity, should be used for lot-to-lot comparisons. Reference standards should be updated as a product evolves but should be finalized by the start of phase 3 trials. Appropriate standard operating procedures (SOPs) should be developed for qualification of a new reference standard.

a. Structural Integrity

A combination of SDS-PAGE, IEF, HPLC, mass spectrometry, or other appropriate physicochemical methods should be used to show that the purified antibody is not fragmented, aggregated, or otherwise modified (e.g. loss of carbohydrate side chains). Side-by-side comparisons of production lots to the in-house reference standard should be performed.

b. Specificity

Assays should provide evidence that the binding of the mAb to the target antigen is specific. Once the specificity of the antibody is characterized, it should be screened for cross-reactivity with human tissues (see Section III.A.). The following are some suggestions on the design of specificity studies:

i. Direct binding assays should include both positive and negative antibody and antigen controls. At least one isotype-matched, irrelevant (negative) control antibody should be tested. Negative antigen controls should include a chemically similar, antigenically unrelated compound, if available (e.g. similar chemical nature, size, charge, and charge density).

ii. Whenever possible, the protein, glycoprotein, glycolipid, or other molecule bearing the reactive epitope, should be biochemically defined, and the antigenic epitope, itself, determined. If the antigenic determinant is a carbohydrate, the sugar composition, linkage, and anomeric configuration should be established.

iii. If possible, fine specificity studies using antigenic preparations of defined structure (e.g. oligosaccharides or peptides) should be conducted to characterize antibody specificity by means of inhibition or other techniques. For complex biological mixtures, the lots of test antigen and/or inhibitors used for direct binding tests should be standardized. Inhibition of antibody binding by soluble antigen or other antibodies should be measured quantitatively.

iv. Once the specificity of an antibody has been determined, it is important to quantitate antibody binding activity by affinity, avidity, immunoreactivity, or combinations of these assays, as appropriate. A number of published methods are suitable for measurement of antibody binding activity (9, 10).

c. Potency Assays and Potency Specifications

Potency assays are used to characterize the product, to monitor lot-to-lot consistency, and to assure stability of the product. Potency may be measured by a binding assay, a serologic assay, activity in an animal model, and/or a functional assay performed *in vitro* or *in vivo*. It is desirable that the assay(s) bear the closest possible relationship to the putative physiologic/pharmacologic activity of the product and be sufficiently sensitive to detect differences of potential clinical importance in the function of the product. In particular, when the performance of the antibody depends not only upon antigen binding but also on other critical functions, it is desirable that the potency assay(s) measure all such functions.

Documentation of the potency assay's performance, including sensitivity, intra- and inter-assay variation and robustness, should be provided.

i. Antibody binding activity may be quantitated by ELISA, RIA, radioimmune precipitation, cytotoxicity, flow cytometry, or any other standard, appropriate method. Activity should be expressed as specific antigen-binding units per mg or µg of antibody. Product should be compared to an in-house reference standard. Appropriate measurements of antibody affinity, if established, may be a useful adjunct to other assays. Parallel line bioassay or a similar, valid statistical procedure should be used in calculating potency.

ii. The potency of a mAb may also be tested by measurement of *in vivo* function in animal models, although such assays are often cumbersome and difficult to standardize and should not be the sole measure of potency.

iii. The permissible range of values in potency assays that reflects adequate biological activity of a product should be based on experience with a particular antibody. Ideally, potency assays should be correlated with *in vivo* activity in order to develop control tests which will ensure an effective product. This implies that multiple production lots should be used during the clinical development program and potency assay results should be correlated with clinical performance. When clinical performance is measured by *in vitro* tests used as surrogates of efficacy, such tests should be validated in a phase 3 clinical trial of appropriate design.

6. Anti-idiotype vaccines

The following issues should be addressed for anti-idiotype vaccines:

a. In the case of an anti-idiotype vaccine (Ab2 vaccine), the Ab2 immunogen should be characterized as to the Ab2 type, e.g. classical type (Ab2β) or antigen mimic (Ab2α) (11).

b. Ab2α vaccines should be shown to be reactive with the appropriate population of human Ab1 (antibody to nominal antigen) if such antibodies are available.

c. The Ab2 preparation should be studied for the appropriateness of response (to target antigen) in xenogeneic as well as syngeneic animals (12).

7. Monoclonal antibodies conjugated with toxins, drugs, radionuclides or other agents (immunoconjugates)

Immunoconjugates are typically produced by chemical processes using specific reagents to link the unconjugated antibody with a non-antibody agent. Alternatively, immunoconjugates can be obtained as chimeric recombinant proteins containing non-immunoglobulin and immunoglobulin sequences in the same polypeptide chain. In addition to previously discussed recommendations for unconjugated (naked) mAb, manufacturers of immunoconjugates should address the following:

a. Construction of the Immunoconjugate.

A full description of the reagents and the process used to construct an immunoconjugate should be submitted, including:

i. A description of components such as toxins, drugs, enzymes, and cytokines that are linked to

the mAb, including: the source, structure, production, purity (including demonstration of freedom from adventitious agents), and characterization of all components (if components are purchased, a certificate of analysis should be supplied).

ii. A description of chemical components, such as linkers and chelating agents, that will be used in preparing the immunoconjugate. These should include documentation of the sources of reagents and method of preparation and determinations of residual impurities from synthesis or purification. Charts of the synthetic reaction pathways and any relevant published or in-house data concerning the toxicity of chemicals used in the production of an immunoconjugate should be provided.

iii. The average ratio of coupled material to antibody and the number of conjugated moieties per antibody should be determined as the first step in establishing lot release criteria for the final product and developing the relationship between immunoglobulin substitution number, potency, and stability.

iv. Products prepared using recombinant DNA technology (e.g., derived from transfected cell lines or microbial cell substrates, chimeric, reshaped, complementarity determining region [CDR] grafted, single chain Fv antibodies, and recombinant immunoconjugates) should follow recommendations discussed in references 1-7, as appropriate. The stability of recombinant immunoconjugates should be studied carefully, as such chimeric proteins may have altered conformational stability, solubility or tendency to aggregate compared to their component polypeptides in their native structures. Loss of specific immunoreactivity due to denaturation or formation of aggregates (e.g. "diabodies" formed by recombinant Fvs) may lead to altered pharmacokinetics and/or binding to non-target tissues.

b. Purity of the Immunoconjugate

i. Special care should be taken to ensure that the antibody preparations are as free as possible of extraneous immunoglobulin and non-immunoglobulin contaminants as such contaminants could react with nuclides, toxins or drugs during the construction of the immunoconjugate.

ii. The amount of free antibody and free components in the final product should be determined with limits set for each. Reactive intermediates should be inactivated or removed.

c. Immunoreactivity, Potency and Stability of the Immunoconjugate

Coupling of toxin or drug to an antibody may alter the activity of either component.

i. Immunoreactivity before and after coupling should be assessed using appropriate methodology (9, 10).

ii. Activity of the non-immunoglobulin component of immunoconjugates, should be assessed by a potency assay whenever appropriate (e.g., toxins, cytokines or enzymes, but not radio-immunoconjugates intended for use in imaging)

iii. Limits on the percent change in immunoreactivity resulting from construction of the immunoconjugate should be established as part of product specifications.

iv. The immunoconjugate should be tested for stability *in vitro* by incubation in pooled human

serum at 37° C under sterile conditions. Plasma may be used instead of serum, provided that the anticoagulant used does not affect the stability of the immunoconjugate (e.g., chelating agents may react with some radioisotopes, heparin may interact with basic proteins, etc.). Aliquots should be analyzed at timed intervals for the concentration of intact immunoconjugate and degradation products. The conditions under which product stability is evaluated and the positive and negative controls used should be fully described. Stability in human serum or plasma is not relevant for topically administered immunoconjugates which are demonstrated not to be absorbed into the bloodstream. It should be established whether or not such immunoconjugates or their components are detectable in plasma after topical administration, and whether or not they elicit an immune response.

d. Specific Issues Related to mAb Coupled to Radionuclides

The preparation of the radioimmunoconjugate should be performed in a standardized, well-controlled, and validated manner. Methods should be developed to estimate the percent radioactivity in each of the three species of concern: free isotope, conjugated mAb, and labeled, non-mAb substances.

i. It is recommended that the initial IND submission for a radiolabeled mAb contain analytical results from two to three radiolabeling runs that demonstrate the preparation of an immunoreactive, sterile, and pyrogen-free product. These radiolabeling runs should be performed by the same personnel who will radiolabel the mAb for the study, using the reagents that will be used for the study.

ii. Radiopharmaceutical grade isotopes should be used when preparing immunoconjugates. The sterility and pyrogen-free nature of each isotope should be documented by submission of a certificate of analysis and letters of cross-reference for manufacturing information.

iii. The concentrations of covalently-bound and free isotope in the final product as well as residual levels of labeling reagents and their decomposition products should be determined during the trial labeling runs.

iv. Quality control tests that will be performed before and/or after each patient administration should be described.

v. When appropriate, colloid formation by the radio-immunoconjugate should be determined and limits set for it.

C. QUALITY CONTROL AND PRODUCT TESTING

1. Cell line qualification

Qualification of the cell line for production of a mAb to be used as a biologic therapeutic should include screening the master cell bank (MCB) and the working cell bank (WCB), at least on a one-time basis, for endogenous and adventitious agents utilizing the tests outlined in Table I and described in the Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals (1). Because the WCB is derived from the MCB and propagated for only a few additional passages in tissue culture, abbreviated testing for detecting newly introduced contaminants is acceptable. Any virus contaminant should be quantified and, wherever possible, identified in order to establish the extent of virus clearance that the purification process should achieve (see also Section II.C.5.). The tropism of virus contaminants for human cells should be determined by appropriate infectivity assays. In the case of tissue culture or fermenter production, end-of-production cells (EPC) should be tested at least once to evaluate whether

new contaminants are introduced or induced by the growth conditions. EPC should also be re-examined when there are changes in culture medium or in the scale of production. Cells at the limit of *in vitro* age used for production can be tested in lieu of EPC to allow for possible extensions in the length of time cells are kept in culture as manufacturing schemes are developed. The term EPC will be used throughout this text for ease of consultation. For cell lines which are known not to be susceptible to infection with mammalian viruses (e.g. plant cells, some insect cells), bacterial and fungal sterility testing, and in some cases testing for Mycoplasma or other mollicutes such as Spiroplasma, will be the most important concerns. Consultation with CBER is advised before using these cell substrates.

Table I
Cell Line Qualification

Tests	MCB	WCB	EPC
Sterility	+	+	+
Mycoplasma	+	+	+
Virus			
Adventitious	+	-	+
Species-specific*	+	-	-
Retrovirus[Ω]	+	-	+
Authenticity	+	+	+

* Tests for rodent, primate, or human viruses (other than retroviruses), as appropriate
ΩRetrovirus testing is not required for murine hybridomas. All other cell substrates should be tested as described in Section II.C.1.d.

a. Cell lines should be free from bacterial and fungal contamination as demonstrated by sterility testing. Recommended testing procedures for mycoplasma (cultivable and non-cultivable) are described in Ref. 1.

b. Screening for adventitious viruses (other than retroviruses) should include routine *in vivo* and *in vitro* tests (1).
c. Screening for species-specific viruses (other than retroviruses):

i. The mouse antibody production (MAP) test for murine cell lines (see Appendix II), the HAP test for hamster lines, and the RAP test for rat lines should be used. *In vivo* testing for lymphocytic choriomeningitis (LCM) virus, including non-lethal strains, is recommended. Testing of hamster cell lines should include minute virus of mice (see III.B.1.c.).

ii. Material that is contaminated with LCM, reovirus, Sendai virus, or Hantaan virus should not be used for mAb production.

iii. Cell lines from non-human primates should be screened for the following: herpes viruses (simiae and SA-8), cytomegalovirus (sCMV), encephalomyocarditis virus, simian hemorrhagic fever virus (SHF), varicella virus of simians (sVZV), adenovirus, SV-40, monkeypox, rubeola, and Ebola virus. Any other zoonotic agents suggested by the cell line derivation history should be screened for.

iv. Human cell lines should be screened for Epstein-Barr virus (EBV), cytomegalovirus (CMV), hepatitis B (HBV) and HCV, human herpes virus 6 (HHV-6), and any other viruses that are suggested by the medical history of the donor and type of tissue used to establish the original line. Cells from patients who are known to have developed Creutzfeld-Jakob disease (CJD) or other TSE, or from persons with two or more genetically related family members with CJD, should not be used.

v. Heterohybridomas using cells from 2 different species should be tested as appropriate for both species of origin.

vi. For cell lines of other species please consult with CBER.

d. Retrovirus testing of cell lines: Retrovirus contamination of cells from different species varies. The following should be considered when designing studies to detect retrovirus:

i. Murine cells used to produce monoclonal antibodies should be considered inherently capable of producing infectious murine retrovirus. The amount of retrovirus in the unprocessed bulk should be quantitated on a series of bulk harvests and shown to be consistent from lot to lot (1). Endogenous virus particle burden should be determined at the end of a typical fermentation, prior to purification, preferably by thin-section EM on material pelleted by ultracentrifugation. Particle burden determination is preferable to infectivity assays at this stage of production because it does not depend upon the susceptibility to infection of the cell lines used for virus amplification and it provides a "worst case scenario" of the level of viral contamination. Thin-section EM also allows morphological observation of viruses. Other, novel methods of equal or superior sensitivity and general applicability may be acceptable, if appropriately validated. Sufficient retrovirus removal by the purification scheme should be demonstrated (see also Section II.C.4.).

ii. Rat myeloma cell lines and hybridomas may not express retrovirus (13).The absence of detectable retrovirus, however, should be demonstrated by co-cultivation of the test article with a cell line(s) susceptible to a wide range of retroviruses combined with a sensitive detection assay, including examination of EPC and several production lots. If retrovirus is not detected by infectivity assays or electron microscopy, further clearance studies may not be needed. It is suggested that purification schemes for mAb produced by rat cell lines include one or more robust retrovirus inactivation or removal step.

iii. CHO cell lines express defective retrovirus particles (14). Whether hamster cell lines express infectious retroviruses has not been shown. Sponsors should demonstrate the lack of infectious hamster retroviruses by means of the most sensitive infectivity assays available. These include co-cultivation of the test article with a cell line(s) susceptible to a wide range of retroviruses combined with a sensitive detection assay. As a product moves into pivotal clinical trials, it may be necessary to make additional attempts to detect potential infectious virus by utilizing a wider range of indicator cells, including human cell lines (15, 16). Because of uncertainty about the validity of infectivity assays for hamster retroviruses, sufficient retrovirus particle removal by the purification scheme should be demonstrated (see also Section II.C.4-5). It is suggested that purification schemes for mAb produced by hamster cell lines include one or more robust retrovirus inactivation or removal step.

iv. Hybridomas or transfected clones produced from cells of non-human primate or human origin should be examined for the presence of retrovirus. Generic assays, such as transmission electron microscopy (TEM) or reverse transcriptase (RT) can be used to assess the presence of retrovirus. Other assays may be used, as long as they are appropriately validated. In addition, all primate cell lines should be screened for simian immunodeficiency virus (SIV), simian T lymphotropic virus (STLV), Foamy virus, human T lymphotropic virus (HTLV), and human immunodeficiency virus (HIV). Cell lines from non-human primates should be additionally tested for presence of simian retroviruses (SRV).

e. Each cell clone generated by stable transfection of widely used parental lines (e.g. CHO) should be considered as a new cell line from the standpoint of viral safety. Such clones may have significantly different genetic characteristics compared to the parental line as a result of the transfection procedure itself, the clonal selection process and positional effects due to random integration of vector DNA into the

cellular genome. Each clone should be screened for retroviruses and for adventitious viruses as described above and in Table I. Screening for species-specific viruses can be done once, on the MCB of the parental line.

f. Authenticity testing should confirm the cell line's species of origin, identity, and lack of cell-line cross-contamination.

2. Lot-to-lot quality control monitoring of unprocessed bulk lots and purified bulk lots, and final product specifications

Quality control monitoring should be performed on each lot of product, as defined in 21 CFR 600.3(x). Table II summarizes lot-to-lot product safety testing.

Table II
Lot-to-lot product safety tests

Tests	Unprocessed Bulk	Purified Product	Final Product
Sterility	+*	+	+
Mycoplasma	+	-	-
Virus			
Adventitious[Ω]	+	-	-
Species-specific[δ]	+	-	-
Retrovirus[ℙ]	+	-	-
Polynucleotide	-	+	-
Endotoxin	-	-	+

*Bioburden testing with acceptable limits is sufficient at this stage.
Ω *In vitro* testing with three indicator cell lines should be performed routinely for non-ascites material*In vivo* testing is generally done once for non-ascites material but should be repeated when production methods change.
δ MAP, RAP or HAP testing for ascites only..
ℙQuantitation of retrovirus (preferably by TEM) in the unprocessed bulk is important for murine hybridomas. For other hybridomas, generic assays for detection and quantitation of retrovirus, such as TEM coupled with appropriate co-cultivation assays are important if MCB or EPC are positive.

a. Unprocessed Bulk Lots

i. There should be set limits for bacterial bioburden in unprocessed bulk material. If bioburden testing of pooled ascites harvests shows the presence of viable contaminants, they should be quantified, and allowable limits for bacterial contamination should be set based on manufacturing experience. The identity of the bacterial species should be determined on a periodic basis and whenever the allowable limits for contamination have been exceeded. Filtration of ascites harvests through a ≤ 0.45 μm filter prior to storage is recommended (see also II.C.2.a.*ii.*).

ii. Tests for cultivable and non-cultivable mycoplasma should generally be performed on

unprocessed bulk hybridoma supernatants, prior to any clarification by filtration (1). The filtration of unprocessed bulk ascites through a 0.45 μm filter followed by storage at ≤-60° C prior to testing for mycoplasma is acceptable if samples of unfiltered material are retained for testing. If mycoplasma contamination of animals or unpurified bulk ascites or hybridoma supernatants is detected, these should not be used or processed further.

iii. In vitro virus testing with three indicator cell lines (e.g. Vero, MRC5, 3T3) should be performed routinely. *In vivo* testing is generally done once (as part of cell line qualification, Section II.C.1) but should be repeated when production methods change (1). Bioreactors containing hamster cells can become contaminated with minute virus of mice that may escape detection in routine *in vitro* assays. MAP testing or PCR testing for this virus appear to be more sensitive. In all cases, the frequency of monitoring should be specified in SOPs and justified based on actual experience when using continuous production in contrast to batch production. When contamination with a particular virus is encountered in a facility, consideration should be given to modifying the routine testing program in order to detect that virus.

iv. Species-specific virus testing should be performed (see Table II and II.C.1.c.).

v. Murine retrovirus contamination should be quantitated routinely for bulk ascites harvests.This may not be necessary if the sponsor can provide data showing that little variation exists in the concentration of mAb and retroviral load of their bulk ascites over several consecutive manufacturing runs, and the purification scheme used can remove substantially more than the highest load observed. If ascites production uses different groups of mice, periodic serologic monitoring for species-specific viruses should be performed on each group prior to their use for producing ascites. For tissue culture harvests, retrovirus contamination should be quantitated on three clinical grade production lots in order to establish the level of virus contamination for the specific cell line and manufacturing process (1) (see II.C.4). This quantitation of retrovirus should be done preferably by generic assays such as TEM or alternatively by sensitive infectivity assays (see also Section II.C.1.d.). Quantitation should be repeated when changes in tissue culture media, duration or scale of culture are made.

vi. For hybridomas of non-murine origin or other cell substrates, see section II.C.1.d. for appropriate assays to determine whether retrovirus is present. In those cases where MCB or EPC are positive for retrovirus, each lot of unpurified bulk should be examined for detection and quantitation of retrovirus by generic assays, such as TEM, coupled with appropriate co-cultivation assays.

b. Purified Bulk Lots (Drug Substance)

In addition to lot-to-lot safety testing summarized in Table II, routine testing on purified bulk lots of unmodified and modified mAb product should include the following determinations (for discussion of immunoconjugates see Section II.B.7.):

i. If a cell bank containing a known infectious agent is used, CBER staff should be consulted before proceeding with development, and clearance studies should be conducted to demonstrate the removal/inactivation of this agent by the mAb purification scheme. Testing for murine retrovirus during clearance studies should employ infectivity assays which detect ecotropic recombinant murine retrovirus (ERV) and the polytropic or mink-cell focus-forming murine retrovirus (MCF). The infectivity assay should be comprised of an amplification period on a cell line sensitive to infection by these murine retroviruses (for example, Mus dunni cells, 17)

coupled with an appropriate indicator assay (for example, PG4 S+L- assay for MCF virus, immunofluorescence assay with appropriate antibodies for detection of ERV). Assays which do not rely on infectivity, such as PCR-based assays, may be substituted, provided they have been validated for sensitivity and specificity, and that their results are correlated with those of infectivity assays. The first consecutive 3-5 lots of purified bulk should be tested to confirm that the contaminant was removed by the purification scheme. For cell lines containing viruses or virus-like particles, the absence of detectable virus should be confirmed for purified bulk by means of suitable methods having high specificity and sensitivity for the detection of the virus in question. For the purpose of marketing authorization, data from at least 3 lots of purified bulk manufactured at pilot-plant or commercial scale should be provided. However, for cell lines for which the endogenous particles have been extensively characterized, such as CHO cells, and adequate clearance has been demonstrated, it is not usually necessary to assay for the presence of non-infectious particles in purified bulk. Should a human infectious agent be identified in the cell bank, every lot of purified product should be tested and consultation with CBER staff is recommended before extensive product development.

ii. Chemical purity including the residual amounts of extraneous animal proteins, e.g., albumin, immunoglobulin or other contaminants in the final product. An SDS-PAGE analysis, under both reducing and non-reducing conditions, of increasing amounts of purified material should be provided. Generally, silver staining methods are more sensitive but less quantitative than Coomassie blue for SDS-PAGE.

iii. Molecular integrity, including the presence of aggregated, denatured or fragmented product.

iv. Immunoglobulin class or subclass, if used as a test of identity.

v. IEF pattern of the antibody (or its heavy and light chains) in each bulk lot with comparison to the in-house reference standard.

vi. Sterility.

vii. Lot-to-lot testing for DNA content, prior to any excipient addition, is recommended as a way to monitor purification efficiency and reproducibility. DNA content in the final product should be as low as possible, as determined by a highly sensitive method. Low cell viability at harvest may contribute to high DNA levels in unprocessed bulk. It is suggested that, whenever possible, the final product contain no more than 100 pg cellular DNA per dose (18). It is suggested that a method with a sensitivity of 10 pg be used to determine DNA levels (2). An appropriately conducted clearance study for DNA removal may be an acceptable substitute for lot-to-lot testing.

viii. Tests for detection and quantitation of potential contaminants or additives (e.g., antibiotics, other media components, host cell proteins, chromatography reagents, preservatives, or components that may be leached from affinity chromatography columns such as protein A). Whenever possible, contaminants introduced by the recovery and purification process should be below detectable levels using a highly sensitive analytical method. However, for many of these potential contaminants, depending upon their potential for toxicity or immunogenicity, an appropriately conducted clearance study may be an acceptable substitute for lot-to-lot testing. For products intended for marketing, at least 3 exhibit lots should be tested to confirm the removal of the contaminant(s) for which clearance studies have been conducted. Such clearance studies for product contaminants may have to be repeated when manufacturing schemes are changed. We

recommend that antibiotics, particularly penicillin or other beta lactams, not be used. However, if they are used, their removal must be demonstrated by an adequate clearance study. The acceptability of trace contaminants that cannot be removed by standard methods should be discussed with CBER prior to the submission of an IND. Pristane, if used in the propagation of ascites fluid, should be shown to be undetectable by a sensitive test.

ix. A brief description of the formulation process and areas to be used for it should be provided. This description should incorporate information on the processing area, support systems, personnel and product transfers in sufficient detail to highlight the leached from affinity chromatography c

Whenever possible, contaminants introduced by the recovery and purification process should be below detectable levels using a highly sensitive analytical method. However, for many of these potential contaminants, depending upon their potential

Veterinary Drug Products (21).

3. Stability of product

Product stability should meet the demands imposed by the clinical protocol. Accelerated stability testing data may be supportive but do not substitute for real-time data for product approval and labeling.

a. A stability testing program should be developed that includes tests for physico-chemical integrity (e.g., fragmentation or aggregation), potency, sterility, and, as appropriate, moisture, pH and preservative stability, at regular intervals throughout the dating period. See also "Guideline for Submitting Documentation for the Stability of Human Drugs and Biologics" (22) and the relevant ICH document (7). For products that are in clinical trials, significant changes that occur during storage should be reported to CBER. For license applications, and preferably prior to each stage of development, stability tests that support the proposed dating period should be performed on the final-filled product, using the container and closure configuration intended for distribution. For biologics license applications, storage of production intermediates (e.g. unpurified bulk, purified bulk) also should be supported by stability testing.

b. Stability tests for assuring biological activity (e.g. quantitative *in vitro* potency assays) should include a manufacturer's in-house reference standard. Whenever possible, a single lot of test antigen (e.g. purified antigen, cells or tissue) should be used throughout the study. A quantitative potency assay(s) should be used to permit a meaningful comparison of activities.

c. Accelerated stability studies, i.e. stability testing after storage at temperatures exceeding routine storage temperatures, may help to identify and establish which tests are stability-indicating. Specific parameters that indicate stability should be monitored by trend analysis on every lot on the stability testing program.

4. General considerations on quantitation and removal of a retrovirus contaminant

The amount of any retrovirus contaminant in the unprocessed bulk product should be estimated. If a retrovirus is detected, any possible or suspected tropism for human cells should be explored (e.g. by co-cultivation assays). This should be followed by demonstration of removal of the contaminant, using the contaminant itself or a representative analogue of the known contaminant (e.g. a model retrovirus) in a model purification system (1).

Unprocessed bulk supernatant concentrates or ascites should be assayed prior to any manipulation other than clarification by low speed centrifugation, unless it can be shown that virus testing would be made more sensitive by initial partial processing. We recommend that retroviruses contaminating ascites or supernatants produced by rodent cell lines be quantitated by TEM of concentrated samples of supernatant. If TEM results are negative, it should be assumed that the titer of retrovirus is equivalent to the lowest limit of detection ($1x10^6$/ml). Ascites and supernatants produced by non-rodent or hybrid cell substrates should be assayed as described (see Section II.C.2.b.*vi.*). Demonstration that retroviruses are removed or inactivated by the purification scheme should occur prior to phase 1 studies, except for mAb intended for use in feasibility trials in serious or life-threatening conditions (see Section II.D.). References 1, 2 and 8, and paragraphs 5 and 6 below, also discuss the design of studies to demonstrate retrovirus removal. The goal of such studies is to demonstrate that the purification process is able to remove substantially more virus than is estimated to be present in a single dose equivalent of starting material.

5. General considerations on the design and interpretation of virus clearance studies

These considerations are provided here solely for guidance purposes in the design of virus clearance (i.e., removal/inactivation) studies.

a. General Experimental Design

The objective of a virus clearance study is to provide a quantitative estimate of the level of virus reduction provided by the removal/inactivation procedures. Thus, the study should be designed, conducted and analyzed in a manner that will provide accurate information to reliably assess the ability of these procedures to remove/inactivate viruses. The study, including virus infectivity assays, should be designed according to good scientific practice to yield data with accuracy and precision that are amenable to statistical analysis. Each clearance study should include an appropriate control experiment performed in parallel to the experimental condition to assess virus inactivation caused by experimental manipulation (dilution, concentration, filtration, storage etc.). Any observed difference should be used to adjust the virus reduction/inactivation values for each removal/inactivation procedure. It is preferable that the study be conducted under current Good Laboratory Practices (21 CFR, Part 58).

b. Statistics

Virus clearance studies should be supported by appropriate statistical analysis demonstrating that the study is valid and reliable. Virus infectivity assays used to quantitate the virus titer should be sensitive, reproducible and conducted with sufficient replicates to demonstrate statistical accuracy. Sufficient sample volumes should be tested to ensure that there is a high probability of detecting virus in the sample if present. The experimental variability present in virus titration should be determined in virus infectivity assays and reported using confidence intervals. Within assay variability should be calculated using conventional means (standard deviation, standard error of the mean, etc.). Between assay variation can be monitored by the inclusion of an appropriate virus reference preparation run in parallel with unknown samples. The 95% confidence intervals for within assay variation should be established and reported for each virus infectivity assay, and should be in the order of $\approx$ 0.5 $\log_{10}$ of the mean. The quantitative estimate of virus reduction for each procedure should be reported as the reduction factor. This is defined as the $\log_{10}$ of the ratio of the virus load in the starting material to the virus load in the post reduction material ($\log_{10}$ (volume x virus titer of starting material / volume x virus titer of post-reduction material)). In general, this virus reduction factor should be based upon the amount of virus detected in the spiked starting material. The 95% confidence interval for each reduction factor is calculated from the 95% confidence interval of the virus infectivity assays at the beginning (+s) and end (+a) of each procedure using the formula $\approx \sqrt{a^2+b^2}$. Such confidence intervals should be calculated whenever possible in studies of clearance using relevant or specific model viruses (with respect to viruses actually detected in the cell line as opposed to non-specific model viruses). The reduction factors for each procedure are summed to calculate an overall reduction factor for the entire process. Additivity of reduction factors assumes that different steps are independent, i.e. have different mechanisms of action so that virus forms (mutant, aggregates etc.) escaping one step do not have a higher likelihood of also escaping the other. Reduction factors from two steps with the same mechanism of action (e.g. two incubations at low pH) are not necessarily additive. Due to the high intrinsic variability of infectivity assays, reduction factors of one log or less are considered negligible and are not included in the overall reduction factor.

6. Generic or modular virus clearance studies

a. A generic clearance study is one in which virus removal and inactivation is demonstrated for several steps in the purification process of a model antibody. These data may then be extrapolated to other antibodies following the same purification and virus removal/inactivation scheme as the model antibody.

b. A modular clearance study is one that demonstrates virus removal or inactivation of individual steps during the purification process (column chromatography, filtration, pasteurization, solvent/detergent, low pH, etc). Each module in the purification scheme may be studied independently of the other modules. Different model mAb may be used to demonstrate viral clearance in different modules, if necessary. If the purification process of a product mAb differs at any of the virus removal or inactivation modules from the model mAb, this module must be studied independently from the model. The other, identical modules in the procedure may be extrapolated to the product mAb.

c. Applicability: For monoclonal antibodies manufactured at one site, generic or modular clearance data may be extrapolated to other monoclonal antibodies of the same species and H and L chain class and, generally, subclass, derived from the same source (e.g. ascites or tissue culture) and cell substrate. Generic and/or modular clearance will apply only when the mAb have similar biochemical properties and are purified by identical methods. Particular attention should be paid to column elution buffer conditions, including pH and ionic strength, the sequence of columns, protein concentration, dwell times, flow rate, pressure, temperature, and potential problems associated with scale-up, at all steps of virus inactivation and removal. In some cases, sponsors may demonstrate virus removal/inactivation for a particular module at two different values of a given parameter (e.g. ionic strength, dwell time, temperature) and use any values of that parameter falling within this range. In order to apply generic or modular clearance algorithms to a specific product, it is necessary to determine the virus load in the unpurified bulk for each specific product using at least 3 lots (see Section II.C.2.). This information is then used to determine whether the total reduction/inactivation factor provided by the purification procedure ensures that substantially more virus is removed or inactivated than the estimated unpurified bulk titer. For example, generic clearance might apply to a series of murine mAb of different specificities but of the same H and L chain isotypes that are purified in an identical manner, or to a series of humanized mAb of the same H and L chain isotype but with different CDRs, that are purified by identical methods. The concepts of generic and modular virus clearance studies do not apply to products of entirely human origin or to products that have the potential to be contaminated by human pathogens. Consultation with CBER is advised before applying generic or modular virus clearance studies.

d. Virus Clearance Master Files: A sponsor purifying mAb for a variety of applications or a manufacturer producing mAb for a variety of sponsors may submit a Master File containing the data demonstrating virus removal or inactivation for different purification schemes. This Master File may then be cross-referenced in INDs/IDEs or license applications using mAb purified by these schemes if applicability criteria are met.

7. Product testing requirements for mAb used as ancillary products

Testing requirements for mAb used as ancillary products (see I.B. for definition) vary depending on the clinical indication and the stage of product development. From a safety standpoint, they should be characterized in the same way as products intended for *in vivo* administration. However, production steps which follow purification of the mAb can be used as part of the virus removal/inactivation scheme. These include, for example, conjugation of mAb to solid phases for affinity purification, sanitation of affinity columns etc. The concepts of generic and modular clearance studies apply to ancillary products as well. Purity of the ancillary product may not be as critical as for products intended for *in vivo* administrations, provided that the performance of the ancillary product in the production of the final product is acceptable and reproducible, limits are set for impurities and the nature of impurities is known. Leaching of mAb or impurities from the mAb preparations into the final product should be taken into consideration in testing the final product, as appropriate. Removal of mAb or impurities from the mAb preparation may be demonstrated by means of a clearance study. Labeling for the final product may need to carry

precautionary statements about potentially toxic or immunogenic residual impurities.

D. PRODUCT SAFETY TESTING FOR FEASIBILITY CLINICAL TRIALS IN SERIOUS OR IMMEDIATELY LIFE-THREATENING CONDITIONS

1. General considerations

The extent of product testing necessary before a particular clinical trial is initiated depends on the source and nature of the product, the stage of product development and the clinical indication. Abbreviated testing needs described in this Section (II.D.) apply to feasibility clinical trials in serious or immediately life-threatening conditions for which no effective alternative treatment exists. Abbreviated testing does not apply to human products made in human cell substrates, but may apply to recombinant products made from transfected human genes, depending on the cell substrate. Consultation with CBER is strongly advised for sponsors considering the application of abbreviated testing in this setting to products that have the potential to be contaminated by human pathogens. For the purpose of this document, the following definitions should be considered:

> *i*) **Feasibility clinical trials.** These are pilot studies whose objectives include, among others, early characterization of safety and initial proof of concept in a specific patient population. These trials are limited in scope, and are generally conducted in a single center, with a small number of patients (e.g. 5-20). These trials cannot be used by themselves to support licensure of a product. Studies conducted in normal volunteers are not included in this definition.
>
> *ii)* **An immediately life-threatening condition** is "a stage of a disease in which there is a reasonable likelihood that death will occur within a matter of months or in which premature death is likely without early treatment" (21 CFR 312.34).

See 21 CFR 312.34 and the Federal Register vol. 52, No. 99 (May 1977) for a discussion of serious or life-threatening conditions. Application of abbreviated testing requirements to serious conditions which are not immediately life-threatening as defined in 21 CFR 312.34 will depend upon an assessment of the potential risks and benefits to the patient(s). Factors that should be considered in such a risk-benefit analysis include, among others: *i*) the nature and manufacture of the product; *ii*) the nature and severity or stage of the disease; *iii*) the anticipated effect(s) of product administration (e.g., diagnosis, palliation or cure); *iv*) the availability of comparable or satisfactory alternate treatments *v*) characteristics of the patient population (e.g. age, response to previous therapy); *vi*) the number of patients involved and *vii*) the design of the clinical trial (e.g. patient follow-up, safety monitoring etc.). Pre-IND consultation with CBER staff is strongly recommended for sponsors planning to use the abbreviated testing described below for serious but not immediately life-threatening diseases. The guiding principle for these trials is that sufficient information should be provided before testing in human subjects to assure that patients and their contacts will not be put at unacceptable risk. Informed consent issues for these trials are discussed in Section IV.A.1.e. The limited testing described below should not be used to support development beyond the stage of feasibility trials. Therefore, sponsors are encouraged to plan for additional testing and characterization as described in Section II.C. when they intend to pursue advanced clinical development and seek licensure. In designing the purification process, it is advisable to include at least two orthogonal robust virus inactivation/removal steps. This would further reduce the testing necessary to begin initial clinical trials.

2. Product safety data needed before the initiation of feasibility trials in serious or immediately life-threatening conditions

a. Sterility (bacteria and fungi) testing should be performed on the final product. Three vials of final product should be tested. Routine methods in use in the sponsor's hospital accredited clinical diagnostic laboratory can be used for these tests. Mycoplasma and endotoxin testing are strongly encouraged.

b. *In vitro* and *in vivo* testing for adventitious viruses : If the unpurified bulk product is free of adventitious viruses by *in vitro* and *in vivo* tests or if the purification scheme does include at least two orthogonal robust steps (see Table III), these tests are not required.
Table III shows ranges of retrovirus removal (expressed in decimal logs) that might be expected with various robust inactivation/removal steps and is presented to aid manufacturers in the design of mAb purification schemes.

Table III

Inactivation Step	Reported log virus removal
pH ≤ 3.9	**3-4**
heat	**4**
solvent/detergent	**5**
filtration (15-40 nm)	**4-8**

If the mAb is produced as an ascites fluid and the mice used have been MAP tested and found free of species-specific viruses, adventitious virus testing of the final product is not necessary. When testing is necessary, we recommend that a minimum amount of product equal to 3 maximum human doses be used for these tests. For the purpose of these trials, MAP testing can be limited to known human pathogens: Hantaan, LCM, Reovirus and Sendai virus. For mAb produced in primate cell lines or in non-murine cell lines whose potential for contamination by human pathogenic viruses in unknown, the cell lines or the EPC or the unpurified bulk product should be tested as described in Section II.C.1. c and d.

c. Murine retrovirus testing of the final product is needed on the final-filled product only when the antibody is produced in a murine cell substrate and the purification scheme does not include at least two robust orthogonal virus inactivation/removal steps (see Table III). We recommend that a minimum amount of product equal to 3 maximum human doses be used for these tests. Testing of the final product, when necessary, should be done by a highly sensitive infectivity assay, such as amplification in Mus dunni cells followed by detection in PG4 cells or by other sensitive means as outlined in II.C.2.b.*vi.*

d. MAb used as ancillary products (see I.B. for definition) in feasibility clinical trials in serious or life-threatening conditions. Two cases can be distinguished:

i. When the final product can be tested and the results are available prior to administration (e.g. purified recombinant proteins or cells that can be stored frozen), safety testing can be carried out on the final product itself, as determined during review of the final product.

ii. When the final product is administered prior to or without any safety testing and/or processing, testing of the mAb should be performed as described above in paragraphs D.2. a. through c. In this case, amounts of mAb comparable to those used in one run of final product purification should be used.

The same safety considerations apply to complement, DNAase and other biological reagents used as ancillary products for cell depletion in conjunction with mAb.

E. ISSUES RELATED TO MANUFACTURING CHANGES (DEMONSTRATION OF PRODUCT COMPARABILITY)

1. General

Changes in the product manufacturing scheme frequently occur during clinical development of mAb. Sponsors should develop a plan for demonstrating that the products made by the old and new schemes are comparable, particularly when preclinical or clinical data developed prior to the production changes will be used to support further clinical trials and/or marketing applications (23). Similar considerations apply in the case of significant scale up in the manufacturing process (with or without modification of the general manufacturing scheme) implemented during or after completion of phase 3 trials.

When changes in manufacturing occur during early clinical development, plans for evaluation of product comparability should be incorporated into product development strategies. Such plans should be discussed with CBER and, when appropriate, submitted to CBER for review (see ref. 23).

In-process specifications may be affected by manufacturing changes or process scale-up and appropriate revisions should be undertaken. Similarly, process validations (e.g., virus clearance studies, removal of contaminants or leachables) for all affected steps should be repeated after any significant manufacturing change. In the case of process scale-up, it is recommended that, whenever possible, a column geometry and a ratio between sample volume and bed volume as close as possible to that of the original process be maintained. This is particularly important for those steps (e.g., size-exclusion chromatography) where these parameters are critical to the chromatographic process. Ref. 23 should be consulted for more details on demonstrating product comparability.

2. In vitro evidence of product comparability

In general, when a product is obtained by a modified or scaled-up manufacturing scheme, the results of a rigorous physico-chemical characterization and *in vitro* functional comparison (see Product Manufacture and Testing, Section II.B.) will dictate whether additional data (e.g., pre-clinical and/or clinical data) will be needed. A protocol for demonstrating physico-chemical, immunochemical and biological comparability of two products should prospectively define acceptable variation in the results of individual assays and acceptance criteria for product comparability. For quantitative assays (specific biological assays) accurate estimates of inter and intra-assay variations should be provided. Assays with high intrinsic variability are poorly suited for the evaluation of product comparability. Comparisons should test a number of separate product lots in parallel in order to demonstrate the reproducibility of the new manufacturing scheme. An *in vitro* biochemical characterization of mAb comparability should include a side by side comparison of the two products by a number of different techniques. Properly stored retention samples from previous lots should be used for such side by side comparison. A list of techniques could include SDS-PAGE under reducing and non-reducing conditions, Western blot, size-exclusion analytical chromatography, reverse phase high performance liquid analytical chromatography, isoelectrofocusing, mass spectrometry, an analysis of glycosylation including carbohydrate content and composition, peptide mapping or other appropriate tests. *In vitro* functional comparison should include assays aimed at the characterization of the biological function of the antibody (e.g., binding, cytotoxicity, epitope modulation, etc.). Whenever possible, a comparison of the affinity constants of the two products is highly recommended.

3. Animal studies

Depending on the quality of the data and the type of *in vitro* assays, the nature of the manufacturing change and the types of product differences observed or anticipated, a program of comparative testing (pharmacokinetics, etc.) in appropriate animal models may be considered in lieu of human clinical data when biochemical testing shows differences or cannot exclude significant differences in two products. In some cases, pharmacokinetic studies are complementary to *in vitro* studies. Pharmacokinetic studies in animals may be informative, even in the absence of the target antigen, depending upon the question to be addressed and the expected contribution of antigen binding to the biodistribution of specific mAb in humans. The extent of animal toxicity testing that may be needed to assess comparability will depend upon the safety profile of each specific product, the magnitude of the changes in manufacturing, the presence or absence of detectable differences in purity, structure or *in vitro* activity. Sponsors are encouraged to discuss plans for comparative testing of the two products in animals with CBER or to submit proposal for such testing to CBER for review and comment. The proposed program should be appropriate in view of biochemical data and include statistical considerations.

4. Clinical studies to support manufacturing changes

Comparative clinical evaluation of the products produced by different or scaled-up manufacturing schemes may be needed in certain situations:

a. Product activity cannot be adequately characterized by analytical testing.

b. Biochemical or biological testing show differences in the products.

c. Animal testing reveals significant pharmacokinetic or other differences in the products.

d. The formulation of the product has been changed in a way that can affect its bioavailability. The latter changes generally dictate a need for clinical pharmacokinetic studies.

Pharmacokinetic, safety and/or efficacy data may be required depending upon the nature and magnitude of the observed changes in the biochemical and or biological properties of the product.

Additional information on product comparability testing can be found in ref. 23.

III. PRECLINICAL STUDIES

A. TESTING CROSS-REACTIVITY OF MAB

When the same or related antigenic determinant is expressed on human cells or tissues other than the intended target tissue, binding of the antibody to this tissue may be observed. Non-target tissue binding may have serious consequences, particularly when pharmacologically active antibodies or cytotoxic immunoconjugates are used. Accordingly, cross-reactivity studies with human tissues (or cells if applicable) should always be conducted prior to phase 1 to search for cross-reactions or non-target tissue binding. In the special case of bispecific antibodies, each parent antibody should be evaluated individually, in addition to testing of the bispecific product.

1. *In vitro* testing for cross-reactivity

Human cells or tissues are presently surveyed immunocytochemically or immunohistochemically. Appropriate newer technologies should be employed as they become available and validated.

a. Reactivity of the antibody or immunoconjugate should be determined with the quick-frozen adult tissues listed in Appendix I. Surgical samples are preferred. Post-mortem samples are acceptable with adequate tissue preservation. Tissues from at least three unrelated human donors should be evaluated in order to screen for polymorphism. The effect of fixatives on tissues that are known to be positive should be evaluated to ensure that the target antigen is preserved during tissue processing.

b. In special situations it may be appropriate to assay cross-reactivity on representative cultured cell lines, stem cells, and embryonic/fetal tissue.

c. Several concentrations of the product should be tested. The ability to detect cross-reactions may depend on antibody concentration. Antibody affinities as well as expected achievable peak plasma concentrations should be considered when choosing the proper concentrations for tissue binding studies. An "ideal" concentration for these studies may be the lowest mAb concentration that produces maximum (plateau) binding to the target antigen. An attempt should also be made to compare the ratio of specific binding to target tissue to specific binding to cross-reactive tissue. Because non-specific as well as Fc-mediated binding may be observed, it should be distinguished from specific cross-reactions using inhibition assays with purified antigen, when available.

d. Positive and negative controls are essential for interpreting study results. Controls confirm the acceptable condition of the tissues and adequacy of the assay. Anti-transferrin receptor mAb may be a useful positive control, since transferrin receptor is a common and abundant molecule on the surface of growing normal and tumor cells.

e. If a conjugated, chemically modified antibody or antibody fragment is to be used clinically, it should be tested in that form if at all feasible. The substitution of antibodies of similar specificity for cross-reactivity testing is discouraged.

f. When cross-reactions are encountered and there is a reason to suspect genetic polymorphism of the target antigen, studies should be expanded to a larger panel of tissues to better characterize this polymorphism.

g. A comparison of *in vitro* cross-reactivity in tissues from different species is important in determining the most relevant animal for subsequent toxicology studies.

2. *In vivo* testing for cross-reactivity

Cross-reactivity of a monoclonal antibody with non-target human tissues should dictate a comprehensive *in vivo* investigation in animals, when appropriate models are available. This finding, particularly with cytolytic immunoconjugates or antibodies with ADCC activity, generally indicates the desirability of more extensive preclinical testing, including studies in more than one animal species over a range of doses and repeat dose animal studies. Localization to non-target tissues should be kept in mind when designing clinical trials.

B. PRECLINICAL PHARMACOLOGY AND TOXICITY TESTING

1. General considerations

a. Preclinical safety testing of mAb is designed to identify possible toxicities in humans, to estimate the likelihood and severity of potential adverse events in humans, and to identify a safe starting dose and dose escalation, when possible. Preclinical testing concerns surrounding mAb products include their immunogenicity, stability, tissue cross-reactivity, and effector function(s). Species differences may complicate the design and interpretation of preclinical studies. CBER recognizes that animal models expressing the antigen of interest or a closely related, highly cross-reactive epitope are not always available. Pharmacokinetic and pharmacodynamic properties of mAb that are dependent upon specific antigen binding may not be evident in animal studies conducted in species which do not express the antigen of interest. In some cases, xenograft models can be developed by introducing cells expressing the antigen of interest into immunodeficient mice (e.g. SCID or nude mice). Such models can provide information on specific targeting of desired cells, especially with radiolabeled mAb or immunoconjugates. Transgenic models expressing the antigen of interest are another possibility, if available. Whenever they are available, parallel models which explore the effects of mAb against the animal homolog of the antigen of interest can be informative. *In vivo* activity models have proven valuable in providing data which support a rationale for the proposed product use and in defining safety and toxicity. Animal disease models are available to study the effects of mAb on many inflammatory and autoimmune diseases, and allograft rejection. The extent of preclinical safety testing and the results of such testing will influence safety considerations for initial clinical trials (e.g. starting dose, dose escalation scheme, etc.).

b. Preclinical testing schemes should parallel to the greatest extent feasible those anticipated for clinical use with respect to dose, concentration, schedule, route, and duration. The range of doses selected for study should include at least one dose that is equivalent to and one dose that is a multiple of the highest anticipated clinical dose, with appropriate adjustments for interspecies differences in body size. A broad dose range should be explored. The highest doses tested should elicit adverse effects, whenever possible. Dose ranges are best established with a minimum of three doses. The linearity and overall shape of the dose response curve should also be defined by investigation of several doses and dosing intervals. If changes in manufacturing and/or formulation are made subsequent to conduct of preclinical studies, the decision to repeat some or all preclinical studies should depend on an assessment of the impact or likely impact of these changes on the product (see Section II.E.).

2. Animal toxicology studies

When planning toxicity testing for mAb, the following should be considered:

a. If the test article is an unconjugated antibody and there is no animal model of disease activity or animal that carries the relevant antigen, and cross-reactivity studies with human tissues are clearly negative, toxicity testing may not be necessary.

b. When a relevant animal model is available, an attempt should be made to study the dose-dependence of pharmacodynamic effects. The use of a broad range of doses, including high doses may allow a better prediction of the therapeutic index.

c. The properties of a relevant antigen in the animal should be comparable to those in humans in biodistribution, function, and structure. For example, studies of $CD34^+$ progenitor cells in the baboon are useful because the same cell fractions in both species express the $CD34^+$ antigen and produce hematopoietic engraftment. Absolute equivalence of antigen density or affinity for the mAb, however, is not necessary for an animal model to be useful. Differences in binding, for example, may be compensated for by alterations in the dose or dosing frequency. Differences between the animal and human in antigen number, the affinity of a mAb for the antigen, or the cellular response to mAb binding, should be identified. This will allow more accurate extrapolation of safe human starting dose and

estimation of the margin of safety.

d. Routine assessments of mutagenicity are not generally needed for mAb.

e. Reproductive and developmental studies including teratogenicity in an appropriate animal species should be carried out in instances in which the product is intended for repeat or chronic administration to women of childbearing potential. Results of such peri- and post-natal developmental studies should be submitted for marketing approval. Evaluation of male fertility, when appropriate, should be completed before phase 3 trials.

3. Pharmacokinetics and pharmacodynamics

A pharmacokinetic model may aid in the interpretation of preclinical activity and toxicity, and in the recommendation of an appropriate dosing regimen and thereby improve the design of clinical trials. Such studies should aim at determining pharmacokinetic and pharmacodynamic endpoints. Of particular importance to the selection of clinical dosage is determining the relationship activity to area under the curve (AUC) of tissue or blood concentration over time. In considering the relationship of activity to AUC, factors related to the pharmacodynamics of the monoclonal antibody should be used in evaluating potential clinical effects. These factors include pathophysiologic status, threshold for effects as well as molecular events like the rates of association and dissociation for the site of action. Studies of biodistribution may provide the initial evidence for inappropriate tissue targeting by a mAb or explain toxicities that are observed in animals. Interpretation of data should consider species of origin, isotype, whether the mAb is an intact immunoglobulin, a fragment or an immunoconjugate, method of labeling, stability of the immunoconjugate, level of antigen expression in the recipient, binding to serum proteins, and route of administration. Even if antigen is expressed in an animal model, the mAb may bind the human target antigen and its animal counterpart with different affinities. MAb half-life may also be affected by glycosylation, susceptibility to proteases, presence of circulating antigen, and host immune response. The presence of antibodies to the product may alter biodistribution and elimination. In some cases, informative pharmacokinetic studies may be obtained in animal models which do not express specific antigenic determinants, depending upon the role played by antigen binding in product biodistribution, biotransformation and excretion.

a. Selection of the animal species for pharmacokinetic and pharmacodynamic testing should be guided by the following considerations:

> *i.* Preference should always be given to study of a mAb in an animal model in a species that shares a cross-reactive or identical target antigen with humans, whenever such a species is available. For unconjugated mAb directed at human antigens not expressed in animal models or foreign antigens (bacterial, viral, etc.), studies in animal species lacking the target antigen may not be necessary unless they are designed to address manufacturing issues (see Section II.E. on Product Comparability).
> *ii.* Study of non-human primates is appropriate for unconjugated mAb when there are antigen binding data that indicate that primates are the most relevant species.
>
> *iii.* Normal rodent and murine xenograft models should be critically evaluated for their likelihood of predicting accurately human pharmacokinetic behavior of mAb. Xenograft models may be more useful in evaluating the ability of mAb to bind to human tumors *in vivo*.

b. Changes in manufacturing or formulation may result in significant changes in biological activity. Therefore, it is recommended that the material used in the preclinical studies be manufactured using the

same procedures as used or intended for use in manufacturing material for clinical trials. In some cases it may be appropriate to modify the components of the formulation for preclinical testing. For example, substitution of the homologous animal serum albumin for human serum albumin that is used as a carrier will prevent the formation of anti-albumin antibodies in animal studies and thereby increase the relevance of preclinical testing.

c. Pharmacokinetic parameters should be defined using one or more assay methods (e.g., a radiolabeled mAb should be assayed by ELISA and by measurement of radioactivity). In the case of immunoconjugates of any type, intact conjugate should be distinguished from free mAb and free ligand (e.g. toxin, drug, or radionuclide). Pharmacokinetic parameters that are most important for product characterization, as well as most useful for determining product comparability include T_{max}, C_{max}, $T_{1/2}$ and AUC.

d. The development of anti-immunoglobulin antibodies greatly complicates study and interpretation of the effects of repeat dosing in animals. Murine antibodies are non-immunogenic in mice but are immunogenic in humans, making it difficult to extrapolate the results of repeat dose studies in mice to planned repeat dose administration in humans. The reciprocal problem will occur with fully human, chimeric or "humanized" mAb. Repeat dose studies in rodents in this case may be of little value.

4. Preclinical *in vivo* studies with immunoconjugates

a. Immunoconjugates should be tested for stability *in vivo*.

i. Individual components of an immunoconjugate should be measured during pharmacokinetic and tissue distribution studies in animals and compared to the distribution of unconjugated antibody.

ii. The target tissues for the various components and the potential toxicities that they may cause should be established.

b. Immunoconjugates containing radionuclides, toxins, or drugs should undergo animal toxicity testing even when the target antigen is not present in an animal species, because of possible conjugate degradation or activity in sites that are not the result of mAb targeting. Depending upon the nature of the components of the immunoconjugate and the stability of the conjugate itself, separate studies of the components may be warranted. The toxicity profile of each component should adequately describe the incidence and severity of possible adverse effects. Results should be correlated closely with studies of conjugate stability. Studies of the immunoconjugate should be performed in a species with the relevant target antigen or disease model, whenever available and generally in rodents if a target antigen-positive species is not available. Toxicity testing of free toxin or nuclide may be performed in a different species.

c. For immunoconjugates containing radionuclides:

i. Animal biodistribution data may be used for initial human dose estimation.

ii. Animal models that express the targeted antigen, whenever such models are available, are more likely to reveal the effects of antigen "sinks" or tissues with unexpected antigen expression on biodistribution and/or toxicity.

iii. Xenograft models may evaluate tissue targeting and antigen non-specific radioimmunoconjugate distribution problems, but are not helpful at identifying areas of normal

tissue cross-reactivity.

iv. An adequate number of animals should be studied to achieve radiation dose estimates with an acceptable coefficient of variation (usually less than 20%).

v. There should be complete accounting of the metabolism of the total dose of administered radioactivity and an adequate number of time points to determine early and late elimination phases.

vi. Radioimmunoconjugates should be tested for stability *in vitro* by incubation in serum or plasma (see Section II.C.7.). Methods should be developed to estimate the percent radioactivity in each of the three species of concern: free isotope, conjugated mAb, and labeled, non-mAb substances.

IV. CLINICAL STUDIES

MAb administered to humans have usually been well-tolerated. Instances of serious or fatal adverse events have generally resulted from intended or unintended binding of mAb to specific antigens. These events emphasize the importance of screening tissues for mAb cross-reactivity, particularly when relevant-antigen animal models are not available. The results of preclinical tests may alert physicians to potential toxicities and may indicate that more conservative dosing schemes are justified during dose escalation.

A. CLINICAL CONSIDERATIONS FOR PHASE 1 AND 2 STUDIES

1. General

a. Different approaches to Phase 1 and 2 studies may be warranted depending on the nature of the mAb. Initial studies of therapeutic mAb in phase 1 are generally escalation studies of single-doses of the mAb. The goal should be to determine a presumed optimal biologic dose (OBD) that is usually defined by pharmacokinetics or pharmacodynamic measurements (e.g., degree of antigen binding or saturation or target blood levels, determined on the basis of preclinical studies) and, where appropriate, by the tolerability to the agent (e.g., the maximally tolerated dose [MTD]) (24-26). In the case of a therapeutic unconjugated mAb, studies that identify the MTD may not be necessary. Instead, determination of a presumed OBD may be a more appropriate goal. Immune activation, when relevant to the mechanisms of action or toxicity of the mAb, should be evaluated. In the case of radiolabeled therapeutic mAb or immunotoxins, undesired tissue targeting and release of conjugate due to degradation are major safety concerns. Patients receiving immunotoxins should be monitored for capillary leak syndrome and for hepatic, renal, and muscle toxicities.

Some antibody-specific side effects are more likely to occur with certain subclasses of immunoglobulins. These antibodies (e.g., human IgG1 and IgG3, mouse IgG2a) are more likely to activate complement or activate antibody-dependent cell-mediated toxicity (ADCC) via their Fc regions, leading to lysis of bound cells. MAb may also cause desired or adverse effects by blocking or inducing functions of target cells (e.g., cytokine release syndrome following stimulation of T-cell receptors by anti-CD3).

b. In general, subjects in clinical trials of therapeutic and diagnostic products, including mAb, should be representative of the population targeted for eventual product use. Because of the potential immunogenicity of mAb, healthy volunteers may not be appropriate candidates for phase 1 trials. The

nature of the mAb, the target antigen, and the proposed clinical application should be considered before deciding to enroll healthy volunteers in a trial. Situations in which healthy volunteers might be used in early trials include the following:

i. When the risks of studying a new agent initially in the index population are too high, such as when the index population expresses abnormally high levels of antigen (raising specific toxicity concerns) or when the index population may be particularly vulnerable to toxicity because of serious illness or significant organ dysfunction.

ii. When the index population is so ill that safety data are confounded and difficult to interpret.

When healthy volunteers are considered for inclusion in initial studies of a mAb, the informed consent should reflect the absence of direct medical benefit. For healthy volunteers, as for patient volunteers, the informed consent process should also illustrate the potential immediate and long-term risks of receiving xenogeneic proteins. These include possible toxicity, allergic reactions, and, in the case of murine and other non-human mAb, potential future inability to receive or benefit from a diagnostic or therapeutic mAb because of the development of an immune response against the foreign protein.

c. Sponsors and investigators should carefully consider whether single doses of the mAb, multiple doses of the mAb in a single course, or multiple courses of therapy will be most likely to optimize benefit over risk. Concomitant therapies or repeat administration of the mAb may alter its safety and efficacy profiles. Changes in antigen mass (e.g., due to binding and clearance, or to antigen modulation by the mAb) and immune responses to the mAb, for example, may prevent extrapolation of single dose data to multiple-dose schedules. Furthermore, repeat administrations in the face of an antibody response against the therapeutic agent may lead to toxicity and/or loss of therapeutic benefit.

d. Subjects with prior parenteral exposure to xenogeneic proteins or with a history of xenogeneic protein allergies should be excluded from phase 1 studies of mAb products that have been derived from the same or a closely related species.

e. Informed consent issues in feasibility clinical trials in serious or immediately life-threatening conditions:
If applicable, informed consent forms for these trials should clearly state the following in language understandable to the patients:

i. only a limited characterization of the processes used to prepare the product for their ability to remove endogenous or exogenous infectious/toxic agent(s) was performed, and

ii. there may be potential health risks, including hitherto unknown risks, derived from exposure to such agents if they are present in the product.

2. Dose-setting

a. Whenever possible, the selection of the phase 1 starting dose should be based on safety and toxicity information derived from testing in a relevant animal model. When extrapolating from animal doses to human doses, information about the relative affinity of the mAb for the human antigen as compared to its animal analogue may be of great value. The target *in vivo* dose or concentration range should be based both on *in vitro* studies of cells for which antibody-antigen affinity and functional activity (e.g., immune modulation, cytotoxicity) have been measured, as well as on study of a relevant-antigen animal model, if available, to assess *in vivo* activity. If animal studies are judged to be impossible or of no relevance and

initial *in vivo* studies are to be performed in humans, testing should begin at a low dose that is based on extrapolation from tissue culture studies or from available information gathered in clinical trials of a similar mAb.

b. Initial studies of radioimmunotherapeutic mAb should also employ escalating single-doses of the mAb, with the lowest and highest doses based on animal dosimetry and on the projected tolerance of normal organs to radiation. Both the elimination half-life of the mAb and the elimination half-life of the radioactivity should be characterized.

c. If a multiple-dose regimen of a mAb is anticipated, multiple-dose schedules should be explored in late phase 1 or phase 2 trials, after basic data on toxicity, peak levels, clearance, distribution, and biologic effects are available from single-dose studies. The time required for recovery from the biologic effects of single doses (e.g., immune recovery after $CD4^+$ cell depletion or modulation, return of bone marrow function after radioimmunotherapy) should also be well understood prior to initiation of multiple-dose regimens. The rationale for dosing schedules should be provided. The rationale should be based on dose tolerance, available pharmacokinetic and pharmacodynamic data in humans, and on relevant animal models of safety and efficacy. Modified dosing regimens to compensate for a high antibody response against the agent or circulating antigen may need to be studied. Pharmacokinetic studies to determine the relationships of human anti-mAb antibody titers and circulating antigen levels with organ distribution, clearance, and toxicity may be necessary.

d. Before repeat administration of a radioimmunotherapeutic or immunotoxin, the investigator should characterize all organ toxicities and pathology resulting from single dose administration. The timing of recovery from all toxic effects should be determined. Intra-patient dose escalation may confound interpretation of safety data because it may be difficult to determine whether toxic effects (e.g., to bone marrow) are due to prolonged therapy or to increased dosing levels. Intra-patient dose escalation may be appropriate if no toxicity is seen at the initial dose levels or if it is possible to use initial safe "test" doses and if cumulative toxicity is deemed unlikely. If intra-patient dose escalation is performed, consideration should be given to threshold and carryover effects, as well as to the reversibility of clinical and laboratory adverse events.

e. Design of pharmacokinetic studies should include consideration of the species in which the immunoglobulin is produced, the immunoglobulin class and subclass, and the structure of the antibody (e.g., whole mAb, Fab fragment) or immunoconjugate. A relevant study population will have the appropriate antigen and antigen mass. If antigen mass is likely to alter the bioavailability of the mAb, this should be determined in pharmacokinetic studies so that its impact on dose setting and on stratitifcation and analysis of later trials can be considered. Aside from obtaining estimates of common pharmacokinetic parameters, pharmacokinetic studies may also be very useful in situations in which the comparability of different products or formulations is to be determined (see Sections II.E. and III.A.). Pharmacokinetic studies optimally include the following:

i. Determination of plasma concentration profiles, distribution, and clearance of the mAb.

ii. Determination of doses for further study based on dose-concentration effect relationship and correlation with desired concentrations estimated from *in vitro* studies.

iii. Determination of peak and trough mAb levels and elimination rate constants.

iv. Determination of the organs and sites where the mAb is distributed, metabolized, and eliminated.

v. Determination of the fate of immunoconjugates by assaying the whole molecule and its components.

vi. Investigation of the relationships between the elimination rate and the method of administration, antigen load, and presence of a circulating antigen or of an antibody response against the therapeutic agent.

B. IMMUNOGENICITY: CLINICAL CONSIDERATIONS

Monitoring of antiglobulin titers and immune activity is of great importance in evaluating the safety and efficacy of mAb and in designing protocols involving repeat administration. Immune responses to mAb may have little or no effect, or may interfere significantly with the safety and/or efficacy of a mAb.

1. Monitoring the development of antibodies to mAb

Depending on the source of the mAb, assays for anti-immunoglobulins will need to be developed to detect human anti-mouse antibodies (HAMA), human anti-rat antibodies (HARA), human anti-human antibodies (HAHA), human anti-chimeric antibodies (HACA), and anti-idiotype antibodies. As appropriate for the mAb, assays should be developed to detect human immunoglobulins directed against humanized or primatized antibodies, immunonuclides and immunotoxins, their individual components (e.g., ricin), and neoantigens formed by the linked antibody/toxin/nuclide.

a. The timing of sample collection for evaluating the presence of an anti-mAb antibody should take into account whether the mAb is intended to be given as single or as multiple doses. Titers of the anti-mAb antibody should always be established at baseline to account for pre-existing antibodies (including anti-globulin or anti-conjugate antibodies, when appropriate), and also before readministration of the mAb. Post-administration samples may be drawn early (e.g., two weeks after administration), but should also be drawn at later times (e.g., at six-eight weeks).

b. The assay(s) used to detect the anti-mAb antibody should be standardized to the greatest extent possible. Aliquots of a "reference" preparation of antibody, e.g. anti-mouse antibody for a HAMA assay, with defined specificity from a human or primate source should be aliquoted and frozen to facilitate future intra- and inter-study comparisons. The reference preparation can also be used to establish a standard curve for routine testing. The assay(s) should be validated by establishing sensitivity, specificity, precision, and accuracy. Inhibition or competition studies with both negative and positive controls should be used to demonstrate that the assay detects antibodies to the mAb product. Studies should assess the range of reactivity of normal individuals and should evaluate potential interference by serum components such as bilirubin and lipids.

c. The specificity of the immune responses to the mAb should be identified and characterized in a sample of patients. These studies should establish whether the responses are generated against a heavy-chain isotype determinant, a light-chain, constant (C) region, variable (V) region, idiotypic epitope(s), immunoconjugate, or neoantigen. These data will demonstrate whether it is possible to use an anti-mAb antibody test with broad specificity (e.g., detecting human antibodies reactive with antigens of both heavy and light chain constant regions of all foreign immunoglobulin classes), or whether a more restricted anti-mAb antibody test that is idiotype-specific is necessary. In certain instances the anti-mAb antibody assay should include the actual mAb product as the detection antigen.

d. The choice of the appropriate assay for anti-mAb antibody depends on the proposed use and labeling

of the product. Development and validation of the assay should accompany the clinical development of the new mAb. The results of the anti-mAb antibody testing should be correlated with product efficacy and adverse events.

e. A license application submitted for a mAb to be administered in a repeated dosing regimen should include a clinically available, validated test that reliably measures human antibody responses to the mAb, if an anti-mAb response may affect the safety, efficacy or dosing of the product. If a commercially available HAMA (or other anti-mAb antibody) test kit, is available, it may be used provided it has been demonstrated to reliably detect antibody response against the new mAb product. In most cases, humanized mAb require an assay specific for the product itself. If no appropriate anti-mAb antibody test is available, a properly validated test system should be developed by the sponsor.

2. Clinical consequences of immunogenicity

a. When a patient is found to have developed an antibody response against the therapeutic or diagnostic mAb, adverse events should be anticipated and appropriate precautions taken. MAb are generally given in facilities where acute resuscitative care is immediately available. The use of non-hospital settings for mAb administration (e.g., clinic or home) should be justified by clinical safety data. Vital signs should be observed closely for at least one hour after completion of the mAb administration. The possibility of delayed adverse effects from immune responses to mAb should be considered and reflected in the trial design, including appropriate clinical and laboratory testing.

b. Anaphylaxis, anaphylactoid and other immune reactions

i. True IgE-mediated anaphylaxis to whole mouse immunoglobulins is infrequent. It is theoretically possible that anti-human allotype responses of an allergic nature could occur but they have not been reported to be of clinical importance to date. If the mAb is conjugated to chelating agents or toxins, the likelihood of allergic reactions may be greater. In all cases, repeated administration of a mAb increases the likelihood of a hypersensitivity response. Immediate hypersensitivity reactions may range from mild to severe. Skin testing is not advised because it is a poor predictor of sensitivity to mouse immunoglobulins and can cause sensitization.

ii. Infusional reactions such as chills, rigors, aches, and low grade fever, are common during or immediately following mAb administration (the incidence is approximately 5% with several antibodies). The mechanism of these reactions is not clear. The frequency and intensity of such reactions can often be controlled by using slower infusion rates or by pre-medication.

iii. Serum sickness is unusual following mAb administration but has been described. Unlike anaphylaxis and infusional reactions, which occur during or immediately after antibody treatment, serum sickness is delayed by several hours. The correlation between circulating levels of soluble antigen and immunocomplex-mediated adverse events such as serum sickness should be explored if such adverse events are observed.

c. Anti-mAb antibodies can interfere directly with some antibody-based clinical tests for antigens, such as CA-125 and CEA, by binding to the murine detection antibody. Indirect interference with diagnostic assays is theoretically possible if mAb administration induces anti-idiotypic responses that mimic the antigen. When appropriate, evidence for either type of interference should be systematically sought using well designed *in vitro* studies. Ideally, attempts should be made to circumvent such interference and alternative clinical assays should be validated.

d. When subjects are selected for testing a mAb, the risk that future therapy with a monoclonal antibody may be compromised by elicitation of an antibody response against the therapeutic agent or other mAb should be considered and reflected in the informed consent form.

C. PRODUCT-RELATED CONSIDERATIONS FOR PHASE 3 STUDIES

When planning manufacturing changes or scale-up programs during phase 3 clinical trials, sponsors should consider that product comparability may have to be demonstrated (see ref. 23 and Section II.E.). This may or may not require additional clinical studies depending upon the adequacy of preclinical data (see ref. 23 and Section II.E.). Thus, it is suggested that scale-up programs and contemplated changes in product manufacture be anticipated prior to the initiation of phase 3 trials. Sponsors should study a number of separate product lots during drug development to demonstrate that a safe and effective product can be prepared reliably.

D. ADMINISTRATION OF RADIOLABELED ANTIBODIES

1. Dosimetry

Grade 3 and grade 4 organ toxicities have been reported with therapeutic radioisotopes. Therefore, dosimetry estimates for human subjects are required prior to the initiation of phase 1 studies. The dosimetry estimates should be developed with simulation models utilizing an appropriate diagnostic radioisotope label on the antibody. If no diagnostic radiolabel for the antibody is available for simulation, animal studies with the therapeutic radiolabel may be utilized for dosimetry estimates. The actual dosimetry data for the therapeutic radiolabeled antibody, itself, should be acquired concurrent with the initial phase 1 study and reported prior to the initiation of a phase 2 study.

For diagnostic radiolabeled antibodies, as for the therapeutic isotopes, the investigator should provide estimates of the organ dosimetry prior to the first phase 1 study. Final dosimetry calculations from human studies should be completed prior to the submission of the license application.

a. General considerations: Sufficient data from animal or human studies should be submitted to the IND, to allow a reasonable calculation of radiation-absorbed dose to the whole body and to critical organs upon administration to a human subject [21 CFR 312.23(a)(10)(ii)]. See Appendix III for a list of organs to be included in dosimetry estimates.

The amount of radiation delivered by internal administration of radiolabeled antibodies should be calculated by internal radiation dosimetry. The absorbed fraction method of radiation dosimetry is described in two systems [21 CFR 361.1 (b)(3)(iv)]:

i. The Medical Internal Radiation Dose (MIRD) Committee of the Society of Nuclear Medicine

ii. The International Commission on Radiological Protection (ICRP).

The investigator should specify which methodology is used. The mathematical equations used to derive the radiation doses and the absorbed dose estimates should be provided. Sample calculations and all pertinent assumptions should be listed and submitted.

Safety hazards for patients and health care workers during and after administration of the radiolabeled antibody should be identified, evaluated, and managed appropriately.

b. Calculation of radiation dose to the target organ

Investigators should determine the following, based on the average patient:

i. The amount of radioactivity that accumulates in the target tissue/organ.

ii. The amount of radioactivity that accumulates in tissues adjacent to the target tissue/organ.

iii. The residence time of the radioactive mAb in the target tissue/organ and in adjacent regions.

iv. The radiation dose from the radioisotope, including the free radioisotope and any daughter products generated by decay of the radioisotope.

v. The total radiation from bound, free, and daughter radioisotopes associated with the radioimmunoconjugate, based upon immediate administration following preparation and upon delayed administration at the end of the allowed shelf life.

c. Maximum absorbed radiation dose

The amount of radioactive material administered to human subjects should be the smallest radiation dose that is practical to perform the procedure without jeopardizing the benefits obtained.

i. The amount of radiation delivered by the internal administration of radiolabeled antibodies should be calculated by internal radiation dosimetry using both the MIRD and ICRP systems. The higher estimate of the absorbed dose determined from either of these systems should be used in the radiation dosimetry safety assessment.

ii. Because of known or expected toxicities associated with radiation exposure, dosimetry estimates should be obtained as delineated in IV.D.1.a and b.

iii. Calculations should be provided that anticipate changes in dosimetry that might occur in the presence of diseases in organs that are critical in radioimmunoconjugate metabolism or excretion (e.g., renal dysfunction causing a larger fraction of the administered dose to be cleared via the hepatobiliary system or vice versa).

iv. Possible changes in dosimetry resulting from patient to patient variations in antigen mass should also be considered in dosimetry calculations (e.g., a large tumor mass may result in a larger than expected radiation dose to a target organ from a radiolabeled anti-tumor mAb).

v. The mathematical equations used to derive the estimates of the radiation dose and the absorbed dose should be provided. Sample calculations and all pertinent assumptions should be listed.

vi. Calculations of dose estimates should be done assuming freshly labeled material to account for maximum amount of label as well as at the maximum shelf life of the radiolabeled antibody to allow for the upper limit of radioactive decay contaminants and should: (a) Include the highest amount of radioactivity to be administered; (b) Include the radiation exposure contributed by other diagnostic procedures such as roentgenograms or nuclear medicine scans that are part of the study; (c) Be expressed as Gray (Gy) per megaBequerel (MBq) or per millicurie of radionuclide; and (d) Be presented in a tabular format and include doses of individual absorbed radiation for

the target tissues/organs and the organs listed in Appendix III.

2. Early clinical development of therapeutic radiolabeled mAb

a. Evaluations that should be conducted prior to Phase 1 studies.

Prior to phase 1 studies, investigators of therapeutic applications of radiolabeled antibodies should conduct the following evaluations for the average adult to be entered into the study:

i. The therapeutic radiolabeled antibody should be evaluated for *in vitro* stability and composition of the radioactive material to be administered. The expected and acceptable levels of the percent of free radioisotope, the percent of radioisotope bound to immunoreactive antibody, and the percent of bound radioisotope to non-immunoreactive antibody should be established. Calculations of the estimates should be at the maximum planned shelf life of the radiolabeled antibody to allow for the upper limit of radioactive decay contaminants and should be based upon the maximum dose of radioactive material to be administered to patients.

ii. The expected biodistribution and routes of clearance of the administered radiolabeled antibody dose fractions in tissues/organs should be defined.

iii. The expected biodistribution and routes of clearance that might occur in the presence of diseases in organs that are critical in radioimmunoconjugate metabolism or excretion should be described.

iv. The expected biodistribution and routes of clearance that might occur in the presence of immune responses (e.g., HAMA, HAHA, HARA) should be described.

With reference to the radioactive fractions of the administered radiolabeled antibody dose and the patterns of biodistribution, the following issues should be addressed:

v. From the biodistribution estimation, the expected residence time of the radiolabeled antibody fractions in the target tissues/organs and non-target tissues/organs should be determined.

vi. Based on the estimated residence times in each organ, the radiation exposure for each tissue/organ should be estimated.

vii. Based on the radiation exposure for each tissue/organ, the potential toxicity should be described.

viii. Based on the potential radiation toxicity to tissues/organs, toxicity monitoring protocols should be developed and incorporated into the clinical trial.

ix. If the study has increasing doses of radioactive materials (e.g., a study of the maximum tolerated dose), the radiation exposure for tissues/organs and the associated potential toxicities should be estimated for each radiation dose level.

b. Selection of patients for phase 1 trials of therapeutic radiolabeled mAb.

Patients should be entered into phase 1 trials with therapeutic radiolabeled antibodies only after consideration of the following:

i. To reduce the potential for alterations in biodistribution, patients enrolled in early studies should not have prior or concurrent exposure to investigational or approved antibodies.

ii. Patients should be evaluated for immune response to the appropriate species of monoclonal antibody (e.g., HAMA, HARA). Patients demonstrating evidence of immune response should generally be excluded from phase 1 studies.

iii. For proper evaluation of potential adverse events in early studies, patients should be in adequate health to allow follow-up for three months without adjunctive chemotherapy or radiation therapy. Generally, patients should have a Karnofsky score greater than 70.

iv. Consultation with CBER is strongly recommended when considering the inclusion of pediatric patients in early radiolabeled antibody trials.

c. Study design issues for phase 1 studies of therapeutic radiolabeled mAb

Phase 1 studies should be designed to address the following points:

i. The prepared therapeutic radiolabeled antibody should be evaluated for *in vivo* stability. The previously estimated expected and acceptable levels of the percent of free isotope, the percent of bound radioisotope to immunoreactive antibody, and the percent of bound radioisotope to non-immunoreactive antibody should be confirmed and demonstrated to be reproducible. Calculations should be with the routine shelf life of the prepared radiolabeled antibody and based upon the maximum dose of radioactive material administered to patients.

ii. The pharmacokinetics and biodistribution in the patient population should be studied.

iii. The residence times with radiation dosimetry for tumor (when applicable), tissues, and organs should be determined.

iv. The pattern of toxicity, its relationship to administered dose and the organs of concern for acute and delayed radiation injury should be established.

v. Any apparent evidence of response of tumor to the administration of the radiolabeled antibody should be documented.

vi. The trial design should incorporate patient imaging with a diagnostic radiolabel on the antibody to confirm the expected biodistribution and residence times prior to the administration of the therapeutically radiolabeled antibody.

3. Adverse events for patients enrolled in trials of therapeutic radiolabeled mAb

The mechanism for follow-up of patients and reporting of adverse events should be described in the protocol prior to initiation of the trial. Complete evaluation and reporting of the adverse events potentially associated with the therapeutically radiolabeled antibody should be assured. If patients are referred to their attending physicians during the follow up phase, the investigator should plan and control the follow up of the treated patients for complete and timely reporting of adverse events potentially associated with the administration of the therapeutic radiolabeled antibody.

Patients removed from a trial should be continued in follow up for three months. All adverse events

during that time interval should be reported, even if they are not thought to be related to the administered radiolabeled antibody.

4. Clinical development of radiolabeled mAb used as imaging agents

a. Prior to the initiation of phase 3 studies

Investigators of diagnostic applications of radiolabeled antibodies should collect stability, safety and pharmacokinetic information for the average adult expected to be entered into phase 3 studies.

i. The expected percent of free radioisotope, the expected percent of radioisotope bound to immunoreactive antibody, and the percent of the radioisotope bound to non-immunoreactive antibody should be determined. If unlabeled antibody is to be administered in conjunction with radiolabeled antibody, the ratio and amounts of the labeled and unlabeled antibody should be defined.

ii. The biodistribution of the administered radiolabeled antibody dose fractions in tissues/organs should be delineated, and from the biodistribution, the expected residence time of the radiolabeled antibody fractions in the target tissues/organs and non-target tissues/organs should have been estimated. The routes of clearance of the radiolabeled antibody should be determined.

iii. The changes in pharmacokinetics of the radiolabeled antibody with organ impairment, antigen load in the circulation, and tumor burden should be evaluated. The potential for clearance artifact to degrade patient imaging should be explored.

iv. Estimates of appropriate imaging times and techniques should be developed. Adjunctive imaging aids (e.g., enemas, emptying of the urinary bladder) should be evaluated.

v. Evidence of image quality should be gathered. The ability of the radiolabeled antibody to image known and/or occult disease should be documented. These data should be compared to imaging data obtained using standard diagnostic techniques whenever possible.The incidence of false positive localization of the radiolabeled antibody and the incidence of misinterpretation of the images to produce false positive and false negative interpretations should be explored. Disease specific factors (e.g., stage of disease, tumor burden, and co-morbid illness) should be evaluated for impact on technical procedures in the imaging protocol (e.g., time of imaging).

vi. Phase 2 trials should be designed to define the appropriate patient populations for phase 3 trial(s), to define the technical procedures used for imaging in the anticipated patient populations and to identify potential clinical utility of the test to be further explored in later studies.

vii. Multiple clinical sites should be employed in phase 2 studies to assess the reproducibility of the imaging techniques, and of the preparation and administration of the radiolabeled antibody.

b. Pivotal efficacy studies of radiolabeled mAb used as imaging agents

CBER staff should be consulted for review of and comments on Phase 3 protocol(s), prior to the initiation of the phase 3 study(ies). The following elements should be incorporated into each clinical protocol:

i. A prospectively defined and detailed primary efficacy endpoint and analytical plan.

ii. A study population consisting of those patients for whom the imaging agent is intended after licensure. The performance and utility of an imaging test may vary substantially based on the stage, extent, or severity of the disease, determined in part by the results of other diagnostic tests. Therefore, the study population and subpopulations to be analyzed should be carefully defined in terms of stage of disease as well as in terms of diagnostic tests performed and test results prior to study imaging. The protocols should be designed to assess the imaging performance and the utility in the populations.

iii. A plan for acquisition and storage of imaging data for radiolabeled antibody in the confirmatory studies.

iv. A prospective plan for evaluation of imaging performance:

(a). On-site image interpretation and reporting should be defined and documented. To the extent possible, the information available to the on-site reader should be defined by protocol and recorded on the case report forms.

(b). The off-site image interpretation should be the basis of the principal analysis of imaging performance in the phase 3 clinical trial. The off-site image interpretation and reporting of all radiolabeled antibody image findings and all confirmatory imaging should be defined and documented prospectively. The information available to the off-site reader should be defined by protocol and recorded on the case report form. In general, the off-site reader should have little information beyond the entry criteria of the study and specifically should not be aware of the on-site reading, the results of other diagnostic tests, or patient outcome data.

(c). Planned sample size should be sufficient to determine imaging performance measures to a predetermined precision (i.e., 95% confidence interval width). Imaging performance may vary with the stage, extent, and/or site of disease as defined by pre-imaging evaluations, and this should be accounted for prospectively in planning analysis of imaging performance. To determine performance, imaging results should be compared with another indicator of disease, usually results of standard imaging, biopsy, exploration, patient follow-up or some combination of these.

(d). If the planned use of the test is in conjunction with other diagnostic tests, its imaging performance should be determined and reported in groups of patients defined by the results of the other tests. For example, in some cases it will be important to know the imaging performance in patients with positive CT scans and the imaging performance in patients with negative CT scans.

v. A prospective plan for evaluation of clinical utility:

(a).When an agent has a significant incidence of false negative and false positives or significant toxicities, it is particularly important that the clinical utility be assessed to determine whether the value of the diagnostic information outweighs the potential adverse consequences of incorrect information or the toxicities. In this context, clinical utility means the extent to which information obtained by use of the mAb agent in a defined clinical setting can be expected to contribute to outcome, to contribute to the convenience or appropriateness of patient management, or to provide accurate prognostic information.

(b). Based on phase 2 and other data, the protocol should indicate the specific manner in which clinical utility is to be explored. The following issues should be addressed:

(1) The stage and severity of the disease in which the test is to be indicated should be specified.

(2) The protocol should specify whether the test is to be used in conjunction with or in lieu of other diagnostic tests. Because radiolabeled mAb image by a mechanism distinct from that of radiopharmaceuticals or diagnostic devices, the information obtained from a monoclonal antibody image may be complementary to that obtained by those means. For example, a mAb agent may not be as sensitive overall as an accepted standard test, but may be able to detect disease accurately under conditions where the standard technique fails.

(3) How the various results of the test are hypothesized to be clinically useful (for management or prognosis) should be clearly delineated. For example: positive results together with a positive CT scan are sufficiently diagnostic to avoid further diagnostic evaluation including biopsy; positive results are useful to guide biopsy; positive uptake is predictive of response to a specific therapeutic modality.

V. APPENDIX I: NORMAL HUMAN TISSUES USED IN CROSS-REACTIVITY TESTING

1. Adrenal
2. Bladder
3. Blood cells
4. Bone Marrow
5. Breast
6. Cerebellum
7. Cerebral cortex
8. Colon
9. Endothelium
10. Eye
11. Fallopian tube
12. Gastrointestinal tract
13. Heart
14. Kidney (glomerulus, tubule)
15. Liver
16. Lung
17. Lymph node
18. Ovary
19. Pancreas
20. Parathyroid
21. Pituitary
22. Placenta
23. Prostate
24. Skin
25. Spinal cord
26. Spleen
27. Striated muscle
28. Testis
29. Thymus
30. Thyroid
31. Ureter
32. Uterus (cervix, endometrium)

VI. APPENDIX II: MOUSE ANTIBODY PRODUCTION TEST

The following tests for murine viruses (mouse antibody production test (27) should be performed on any MCB and EPC derived from murine cell lines and on all lots of mAb derived from mouse ascites fluid:

1. Ectromelia
2. EDIM
3. GD VII virus
4. Hantaan virus
5. LCM virus, including challenge for non-lethal strains
6. LDH-elevating virus
7. Minute virus of mice
8. Mouse adenovirus
9. Mouse encephalomyelitis
10. Mouse hepatitis
11. Mouse salivary gland (murine CMV)
12. Pneumonia virus of mice
13. Polyoma
14. Reovirus type 3
15. Sendai
16. Thymic virus

VII. APPENDIX III: ORGANS TO BE CONSIDERED IN DOSIMETRY ESTIMATES

1. all target organs/tissues
2. bone
3. bone marrow
4. liver
5. spleen
6. adrenal
7. kidney
8. lung
9. heart
10. urinary bladder
11. gall bladder
12. thyroid
13. brain
14. gonads
15. gastrointestinal tract
16. adjacent organs of interest

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EXHIBIT E

THE MECHANISM OF N-TERMINAL ACETYLATION OF PROTEINS

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I. INTRODUCTION

N-terminal blocking of proteins is a widespread phenomenon in eukaryotes, prokaryotes, and viruses.[1-3] Formyl, acetyl, pyruvoyl, α-ketobutyryl, glucuronyl, α-aminoacyl, pyroglutamyl, murein, glucose,[2,3] and carbon dioxide[4] have been recognized as N^{α}-acylating groups. Some of these blocking groups are extremely rare. The first polypeptide with an N^{α}-acetyl group was discovered in 1958 by Narita.[5] Thereafter, numerous proteins followed. Brown and Roberts provided evidence that about 80% of the soluble proteins from Ehrlich ascites cells are N^{α}-acetylated.[6] Approximately 90% of the proteins from mouse L cells seem, also, to be N^{α}-acetylated.[7] In lower eukaryotic organisms (*Saccharomyces fragilis, Neurospora crassa*), about 50% of the soluble proteins are acetylated.[8] These data demonstrate that N^{α}-acetyl is a very important blocking group.

The blocking nature of the N^{α}-acetyl group becomes apparent during protein sequencing, since the Edman degradation is prevented. The group can only be removed from the N-terminus of a protein under conditions which are harmful to the polypeptide chain itself. The general strategy to solve this problem is to isolate, after enzymatic digestions of the protein, the peptide which contains the blocked N-terminus. Several methods have been used to both detect the acetyl group and the sequence of the blocked peptide,[5,9-12] but nowadays this is most often done by mass spectrometry.[13-15]

Another feature of a protein blocked by an N^{α}-acetyl group is its protection from the action of aminopeptidases. This aspect will be discussed when we describe some current ideas about the function of N^{α}-acetylation. Furthermore, we shall deal with the mechanistic aspects of the attachment of the acetyl group to the protein and the structural characteristics of the N-termini of proteins which are N^{α}-acetylated.

II. WHY ARE PROTEINS N^{α}-ACETYLATED?

It has been suggested that a general function for N^{α}-acetylation is the protection of proteins from proteolytic degradation by aminopeptidases.[16] Experiments to sustain this hypothesis

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have not shown significant differences in the turnover rate of acetylated and nonacetylated forms of feline β-globin[17] and of proteins from mouse L cells.[7] However, these cases have to be interpreted with great care, since the acetylated and nonacetylated proteins were not of the same type. In *Dictyostelium discoideum*, a minor nonacetylated form of cytoplasmic actin with a rapid turnover was found next to a major and more stable acetylated form.[18] However, this turnover is probably not caused by proteolytic breakdown, but rather reflects a precursor-product relationship. For the acetylated form of cytoplasmic actin from cultured *Drosophila* cells[19] and the in vitro acetylated terminal octapeptide of cholecystokinin[20] it has been shown that the acetyl group protects against N-terminal degradation by kidney leucine aminopeptidase and soluble peptidases from the gall bladder, respectively. In the case of the proteins of the eye lens, which have to remain intact during the lifespan of the organ, αA_2-crystallin has an N^{α}-acetyl group which is not essential for protection against N-terminal degradation by lens leucine aminopeptidase.[21] Here the three-dimensional structure of the native protein seems to be a more important factor. Although the N^{α}-acetyl group by its very nature protects against exopeptidases, no decisive evidence has been presented for such a protective role of N^{α}-acetyl groups in vivo.

Although a clear general function for N^{α}-acetylation has not been assessed with certainty, some specific effects for a small number of proteins have been observed. Contrary to normal nonacetylated hemoglobin, feline hemoglobin with N^{α}-acetylated β-chain amino termini is insensitive to the modifying influence on oxygen affinity of organic phosphates.[22] A similar effect has been noted for the human mutant hemoglobin Raleigh (β_1 valine→acetylalanine)[23] and the minor human fetal hemoglobin F_1.[24] N^{α}-acetylation of two of the products from the precursor protein proopiomelanocortin has a profound regulatory effect on the biological activity of these polypeptides: the opioid activity of β-endorphin is completely suppressed,[25,26] while the melanotropic effect of α-MSH is increased.[27] In the first case N^{α}-acetylation is possibly a reversible process. Both acetylated and nonacetylated cytoplasmic actin from cultured *Drosophila* cells participate in the assembly of microfilaments,[19] the latter, however, with less efficiency. A mutant of *Escherichia coli*, in which ribosomal protein S5 is not acetylated, exhibits thermosensitivity.[28] Nonacetylated NADP-specific glutamate de-hydrogenase in a mutant of *Neurospora crassa* is heat-unstable, in contrast to the acetylated form.[29] Moreover, it has been suggested that N^{α}-acetylation is an additional way of acetyl-group handling in the cell,[30] in which case it would resemble other α-amino-group modifications by compounds that reflect high physiological concentrations (e.g., CO_2). This assumption may explain the consistent variation in acetylation during physiological conditions in the yeast alcohol dehydrogenase system, where no effect of acetylation on enzyme activity has been observed.[30]

III. HOW ARE PROTEINS N^{α}-ACETYLATED?

A. At Which Stage?

It was initially thought that different N^{α}-acetyl aminoacyl-tRNAs might serve as the initiators of protein synthesis in various eukaryotic systems[31-33] and that, therefore, N^{α}-acetylated proteins with different N-terminal amino acids should be produced. However, soon it became evident that methionine is the initiating residue of eukaryotic proteins as it is in prokaryotes;[34-37] even on direct search no N^{α}-acetylseryl-tRNA could be detected in rat liver.[38] Moreover, for some N^{α}-acetylated proteins it was shown unequivocally that initiation starts with methionine, e.g., for α-crystallin,[39] ovalbumin,[40] and histones.[41] In many proteins the initiator Met is removed. In *Escherichia coli* the responsible methionine aminopeptidase appears to have a preference for cleaving the Met-X peptide bond if X is Ala or Ser.[42] A ribosomal-bound aminopeptidase has been isolated from this organism which preferentially removes N-terminal methionine from peptides.[43] However, if position 2 is occupied by an

Asp, no hydrolysis takes place. For yeast there is evidence that the methionine aminopeptidase hydrolyzes the Met-X bond very efficiently in cases where X is Ala or Thr, less efficiently if X is Val, and not at all when X is Leu, Ile, or Arg.[44] For immunoglobins and hemoglobin it has been shown that Met-X is cleaved if X is Ala or Val, but not if X is Glu or Asp.[45] These observations clearly demonstrate the important role of the amino acid at position 2. However, the overall conformation of the N-terminal region may, also, be an important factor for removal of the N-terminal Met. It is now thought that for most proteins acetylation takes place at the level of the peptidyl-tRNA, which means that it represents a post-initiational process. It is an enzymatic process that requires acetyl coenzyme A as acetyl donor.

Bloemendal and Strous showed that αA_2 crystallin is acetylated in vitro when a nascent chain of about 25 residues protrudes from the ribosome.[46-48] Acetylation is complete at a length of 50 residues. In ovalbumin, the initiator methionine is removed in vitro when the nascent chain is 20 amino acids long;[49] acetylation takes place at a length of 44 residues. In vitro the β-chain of cat hemoglobin B is acetylated when the nascent chain is about 30 amino acid residues long.[50] That acetylation is a post-initiational process has, also, been suggested for translation of brome mosaic viral RNA in vitro[51] and for rat liver polyribosomes in vitro.[52-54] Acetylation, thus starts when the nascent chain begins to emerge from the ribosome at a length of about 25 residues, or somewhat later if the initiator methionine has to be removed first. Since it is assumed that protein chains are not yet completely folded when emerging from the ribosome, the N-terminal amino acid sequence of a chain may be expected to be an important factor in N^{α}-acetylation.

B. The Enzyme

1. Properties

N^{α}-acetylation is an enzymatic process, and the enzyme responsible has been demonstrated and studied in *E. coli* for ribosomal protein L12,[55] for ribosome-associated proteins from rat liver,[53,54,56] in calf lens,[57,58] rat pituitary,[59-61] ox pituitary,[61] hen's oviduct,[62] and in a ribosomal fraction of wheat germ.[63]

The enzyme from hen's oviduct has been partially purified.[62] It has a pH optimum of 7.2 and a relative molecular mass of about 250,000. Extensive purification was difficult because of the instability of the enzyme. Using N-terminal ACTH-fragments as substrates and acetyl coenzyme A as acetyl donor, this enzyme in vitro preferentially acetylates the α-NH_2 group, as does the enzyme from rat pituitary.[59,60] The latter enzyme is localized predominantly in subcellular fractions sedimenting above 10,000 g; it has a broad pH optimum with a maximum near 7.6 and is very unstable. Glembotski showed that bovine acetyltransferase activity, which is believed to be specific for the formation of α-MSH and N^{α}-Ac-endorphin, is present in the secretory granules from the intermediate pituitary.[61] However, the activity is not membrane-bound. The bovine enzyme has a pH optimum of 7.0. The enzyme from wheat germ is associated with the ribosome fraction and catalyzes the transfer of acetyl groups from acetyl coenzyme A to the N terminus of synthetic des-N^{α}-Ac-thymosin α_1.[63] The pH optimum is below 7.5. The enzyme is activated considerably by KCl concentrations as high as 3.0 *M*. The bovine lens N^{α}-acetyltransferase acetylates amino-terminal ACTH-fragments exclusively at the N terminus using acetyl coenzyme A.[57] The enzyme has a pH optimum near 7.4, an apparent relative molecular mass of 170,000, and an isoelectric point of about 5.[58] This enzyme has been purified 760-fold, although the purification was difficult to reproduce due to extreme instability of the protein.

Acetyl coenzyme A has an apparent Km of 2.2 μ*M* for the rat pituitary enzyme (270 μ*M* ACTH-(1-24)),[60] 8μ*M* for the ox pituitary enzyme (160 μ*M* ACTH-(1-13)-NH_2)[61], and 4.6 μ*M* for the ox lens enzyme (310 μ*M*[Nle^4]-ACTH-(1-10)).[58] These values are roughly 1/10 of the estimated intracellular acetyl coenzyme A concentration.[64]

In conclusion, the nature of the enzymes responsible for N^{α}-acetylation of proteins is still

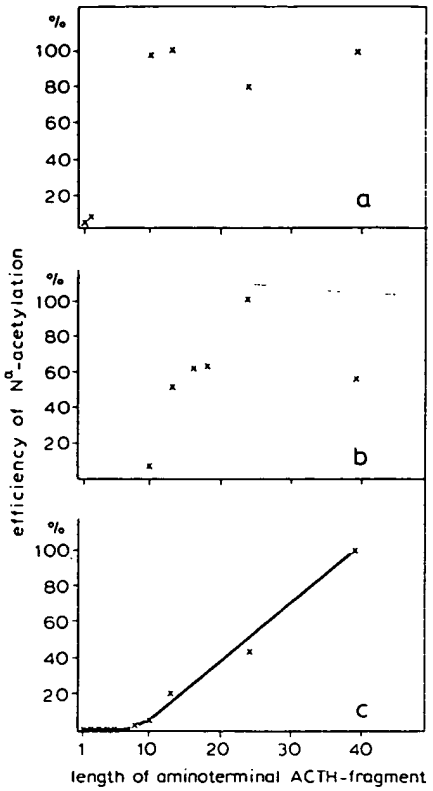

FIGURE 1. Substrate specificity of N^{α}-acetyltransferases from rat pituitary (a), ox intermediate pituitary secretory granule (b), and ox lens (c) with regard to the length of the aminoterminal ACTH-fragments used as substrates. Efficiency is expressed relative to the highest activity measured.

largely unknown. This is due to their low concentration and extreme instability after purification.

2. Substrate Specificity

The substrate specificity of several acetyltransferases has been explored, especially with regard to the chain length of the substrate. The enzyme from rat pituitary[59] needs the N-terminal serine of amino-terminal ACTH-fragments and works equally well with ACTH-(1-10), ACTH-(1-13)-NH_2, ACTH-(1-24), and ACTH-(1-39) (Figure 1a). Free serine and ACTH-(1-2) are not acetylated. Further study corroborated this picture[60] suggesting that the information required for N^{α}-acetylation resides in the first ten amino acids of ACTH-related molecules.

Glembotski[61] carried out extensive kinetic measurements on the enzyme from ox intermediate pituitary secretory granules (Figure 1b). Peptides longer than 18 amino acids showed complex non-Michaelis-Menten kinetics. The presence of the N-terminal Ser-Tyr sequence seems to be required for binding to the catalytic site, but is not sufficient to allow acetylation. Actually a more distant binding site, that needs the residues at approximately position 11 through 18 of ACTH-related peptides, has to be occupied to allow N^{α}-acetylation.

The bovine lens enzyme showed a different behavior compared to amino-terminal ACTH-fragments as substrates[58] (Figure 1c). The length of the fragment is minimally 8 residues. Thereafter, the rate of N^{α}-acetylation increases roughly linearly up to a length of at least 39

Table 1
DISTRIBUTION OF N^{α}-ACETYLTRANSFERASE ACTIVITY IN VARIOUS ORGANS OF THE RAT. RESULTS HAVE BEEN CORRECTED FOR ACETYLATION OF ENDOGENOUS SUBSTRATES IN THE ENZYME PREPARATION[59]

Source	Relative specific activity
Pituitary	100
Lens	18
Heart	11
Lung	24
Kidney	15
Liver	18
Muscle	31
Brain (except pituitary)	41
Serum	1

residues. The reason for this behavior is not known. However, one has to keep in mind that the ACTH-fragments are not natural substrates for the lens enzyme. Replacing the serine at position 1 by the corresponding D-amino acid in ACTH-(1-10) results in a substrate which is not acetylated.[57] The stereochemistry of the first residue is, therefore, a very important factor in N^{α}-acetylation.

The information available at the moment indeed stresses the importance of the first residue and shows that in addition a minimal length of the peptide is necessary.

3. Distribution and Similarity of N^{α}-Acetyltransferases

The distribution of N^{α}-acetyltransferase activity in various organs of rat has been investigated by Woodford et al.[59] (Table 1), using ACTH-(1-10)-decapeptide as a substrate. The extracts of lung, muscle, and brain have a considerable activity, although none is as efficient as that of the pituitary. The lens has a lower specific activity due to its high protein content. It is clear, however, that some N^{α}-acetyltransferase activity is present in all organs but not in serum. Erythrocytes have not been investigated in this study. Several reports have provided evidence that acetylation of proteins can take place in heterologous systems. Berns et al. showed that calf lens α-crystallin A_2 mRNA directs the synthesis of α-crystallin A_2 polypeptides which become acetylated in frog oocytes[65] and in a reticulocyte lysate.[66] Brome mosaic virus RNA produces N^{α}-acetylated coat protein in a cell-free system from wheat germ. The latter system is also capable of acetylating synthetic N^{α}-desacetyl thymosin α_1[63] and rat cytoplasmic actins.[67] Likewise, the reticulocyte lysate gives acetylated cytoplasmic actin of *Drosophila*[19] and *Dictyostelium discoideum*.[68,69] In experiments with isolated N^{α}-acetyltransferases, it has been shown that the enzymes from hen's oviduct[62] and bovine lens[57] acetylate the N terminus of amino-terminal ACTH-fragments, the (1-13)-sequence of which is normally acetylated by pituitary N^{α}-acetyltransferase to produce α-MSH.[61,70] Thus, it is most likely that eukaryotic cells possess one or more N^{α}-acetyltransferases capable of acetylating various proteins with different N-terminal regions. Apparently, they are very similar with respect to substrate specificity.

IV. WHEN ARE PROTEINS N^{α}-ACETYLATED?

In 1975, Jörnvall examined the then known 40 N-terminally acetylated proteins for structural characteristics of the N-terminal region.[16] He established that in the acetylated N-terminal position serine and alanine are predominant as compared with the distribution of N-terminal residues, in general. Branched-chain residues would be more frequently present in the N-terminal region of acetylated proteins. These properties were observed regardless of origin and function of the proteins. Therefore, it seems reasonable to assume that N^{α}-acetyltransferases have very specific requirements as far as the structure of the N-terminal region of proteins is concerned, although the substrate specificity of the enzymes from various tissues and species is very similar. Here we describe in more detail the structural and other requirements for N^{α}-acetylation on the basis of the hitherto known sequences of acetylated and nonacetylated proteins.

A. Compilation of N^{α}-Acetylatled Proteins

The primary structures of proteins as compiled by Dayhoff (1972 to 1978)[1] have been used. Moreover, for N^{α}-acetylated proteins published between 1977 and 1982 the literature has been screened. Only the first ten amino acids were taken into account. Care was taken to check the evidence given for the presence of an N^{α}-acetyl group. Acetyl groups assumed on the basis of homology were allowed if the reference protein had been determined unambiguously. We did not consider an assumed acetyl group of those cases in which the first amino acid is not identical to that of the reference protein. Incidentally, we included a protein which had been decribed in the literature as being N-terminally blocked. This seemed to be reasonable when a homologous N^{α}-acetylated sequence had been reported previously. The complete compilation of N^{α}-acetylated proteins, listing 361 entries, is given in the Appendix. The proteins have been ordered according to their function or origin. No attempts have been made to place homologous proteins together as done by Dayhoff.[1]

When comparing the (1-10)-decapeptide sequences of N^{α}-acetylated and nonacetylated proteins, we used the following seven less stringent rules in order to have available as much structural information as possible.

1. The sequence of at least the first five amino acids must be known. Asx, Glx, or undetermined positions are not allowed.
2. If Asx, Glx, or undetermined positions occur in the second five residues, the sequence up to that residue is considered.
3. For proteins for which identical sequences are known in more species, only one species is taken into account. However, a variant is accepted as a new entry. Shortened sequences, which are N-terminally identical to complete (1-10)-sequences, are not accepted.
4. For proteins in which there is tissue-specificity, gene multiplicity, allelic variation, or species-dependent diversity, every tissue, gene, allele, or species product is considered as a protein of its own.
5. Precursor proteins with the same N-terminus as the product are not accepted.
6. Acetylated proteins with incomplete blocking ($\leq$60%) are not accepted.
7. In the category of nonacetylated proteins all otherwise N-terminally blocked proteins are included.

For the determination of hydrophobicity values we used the "hydropathy" index for amino acids of Kyte and Doolittle,[71] which is based on water-vapor transfer free energies and the interior-exterior distribution of side chains from amino acids. Values vary from -4.5 for Arg through -0.4 for Gly to $+4.5$ for Ile. For every protein the hydrophobicity

was averaged over the number of positions involved (span length) and is, thus, given per residue. Moreover, the hydrophobicity per residue was averaged over all proteins.

In order not to overemphasize structures of which many species variants are known, it was necessary to use some means of selection. For comparison of the N-terminal amino acids only, we used four stringent selection rules:

1. The N-terminal amino acid must be known.
2. In the case of homologous proteins only a single representative sequence is taken into account. However, when there is reasonable evidence for products of duplicated or allelic genes, one product of each orthologous gene is considered.[72] This is the case for tissue-specific proteins, isozymes, and proteins which are only found in a restricted group of species. The product of each unique gene is thus included in our analysis.
3. However, if a species variant has an N-terminal amino acid differing from the reference protein it is taken into account.
4. Precursors which give a product having the same N-terminal sequence are not taken into account.

These rules are not always unambiguous, since it is not known in each case if gene products are orthologous or paralogous. Therefore, incidentally, the decision has been arbitrary. After selection there is still some bias, because of the presence of related gene products.

We estimated the total number and the nature of all sequences known up to 1978 by applying criterion 2 and 4 of the less stringent rules on the cumulative index of Supplement 3 of Dayhoff (1978).[1]

B. The N-Terminal Residue of Proteins, in General

From experiments by Waller,[73] Horikoshi and Doi,[74] and Brown[75] it is known that methionine, alanine, and serine are the main N-terminal amino acids of the soluble unblocked proteins of prokaryotes, and that in eukaryotes alanine and serine are predominant. When all protein sequence data from Dayhoff (1972 to 1978)[1] were assessed for uniqueness with the stringent selection rules, 582 proteins were left. Table 2 lists the N-terminal residues of these proteins and their relative amounts. When Ala, pyrrolidone carboxylic acid (π-Glu), Met, and Ser are taken together, they amount to 46.5% of the total. On basis of the average amount of these amino acids in proteins (Dayhoff, 1978),[1] this should have been around 20%. Obviously, these amino acids are found much more frequently at the N terminus of proteins than expected. This is, also, clearly demonstrated by the ratio between these N-terminal amino acids and the average presence of these residues. Only Ala, π-Glu, Met, and Ser have a ratio higher than 1. Since the protein sequence data set from Dayhoff contains proteins, both soluble and insoluble, from eukaryotic, prokaryotic, viral, and bacteriophageal origin, it is clear that the N-terminal amino acids determined in soluble unblocked proteins, from either eukaryotic or prokaryotic source, as described above, do not have to fit exactly the protein data of Dayhoff. Immunoglobulins, some contractile system proteins, etc. are not present in prokaryotes and lower eukaryotes which were used for the survey by Waller,[73] Horikoshi and Doi,[74] and Brown.[75] However, the predominance of Ala and Ser in the proteins listed by Dayhoff (1972 to 1978)[1] is quite consistent with these determinations.

The relative abundance of Met at the N terminus may be explained by the fact that this probably is the initiator methionine still present in the chain. Considering the genetic origin of the other three, Ala, Ser, and π-Glu (the latter being assumed to be derived from Gln[76]), it appears that Ala is encoded by nucleotide triplets starting with G (4x), Ser from codons with U (4x) and A (2x), and Gln from C (2x). Assuming an equal probability for the use of each codon, we find that there is a 50% probability that the first nucleotide of the codon used for these N-terminal residues is A or G. However, Manderschied et al. reported that

Table 2
N-TERMINAL AMINO ACID OF PROTEINS COMPARED WITH THE AVERAGE PRESENCE IN PROTEINS (DAYHOFF, 1978).[1] THE NATURE OF THE BLOCKING GROUP, IF ANY, IS INDICATED

N-terminal amino acid	Number of proteins	Presence in N-terminal position (%)	Average presence (%)	Presence on N-terminus/average presence	Precentage of N-terminal blocking and nature of the blocking group
Ala	101	17.4	8.6	2.0	3.4 Ac + 0.2 Me
Arg	25	4.3	4.9	0.9	
Asn	10	1.7	4.3	0.4	0.2 Ac
Asp	27	4.6	5.5	0.8	0.3 Ac
Cys	15	2.6	2.9	0.9	
Glu	30	5.1	6.0		
Gln	3	0.5	3.9	1.6	
π-Glu	59	10.2			10.2 π-Glu
Gly	33	5.7	8.4	0.7	0.5 Ac + 0.2 F
His	8	1.4	2.0	0.7	
Ile	18	3.1	4.5	0.7	
Leu	29	5.0	7.4	0.7	
Lys	21	3.6	6.6	0.5	
Met	46	7.9	1.7	4.7	1.2 Ac + 0.3 F + 0.2 Me
Phe	9	1.5	3.6	0.4	
Pro	19	3.3	5.2	0.6	0.2 F + 0.2 Me
Ser	64	11.0	7.0	1.6	4.3 Ac
Thr	24	4.1	6.1	0.7	
Trp	2	0.3	1.3	0.2	
Tyr	9	1.5	3.4	0.4	
Val	30	5.2	6.6	0.2	
	Total: 582	118 Blocked			Total: 21.4%

in mRNA sequences the initiation triplet AUG is very often followed by a codon starting with a purine nucleotide.[77] They found that the formation of *Escherichia coli* 30S initiation complex with initiator-tRNA occurred far more readily with the tetranucleotides AUGA and AUGG than with AUG itself or AUGU. This suggested that the initiator-tRNA, which has a U-residue following the anticodon, recognizes the initiation site through four nucleotides. In a search for the information necessary for an optimal binding of an mRNA to *E. coli* ribosomes, it was found that in the optimal fit an A follows the AUG codon.[78] However, the mRNA sequences showed that actually about 70% of the nucleotides following AUG is A or G. It is not known if these rules are also valid for eukaryotic mRNAs. For many proteins the initiator Met is removed, especially if the second residue is Ala or Ser.[42,45] In most cases the N-termini of proteins correspond with the second codon of the mRNA involved. If the hypothesis of Manderschied et al. had general validity, N-terminal Ala and Ser would fit reasonably well. However, it does not give an explanation of why the other amino acids encoded by triplets starting with an A and G are present much less frequently at the N-terminus of proteins than are Ala and Ser.

Upon examination of known N-terminal residues it appears that 21.4% are blocked, either by a formyl (0.7%), a methyl (0.6%), a pyroglutamyl (10.2%), or an acetyl group (9.9%). Of the 21.4% blocked N-terminal amino acids 19.8% are represented by just four species: Ala, π-Glu, Met, and Ser. Apparently, there is a strong preference for N-terminal blocking of exactly the same amino acids which are overrepresented at the N-terminus. No explanation for this phenomenon can be given at the moment.

C. Factors Which May Influence N^{α}-Acetylation

Several factors may influence N^{α}-acetylation of proteins. For instance, the distribution of the enzyme within a tissue is very important, as has been demonstrated for the products of the precursor protein proopiomelanocortin. ACTH, which has N-terminally the same sequence as α-MSH, is not acetylated, while α-MSH mostly is.[1] α-MSH is acetylated in *pars intermedia*, whereas it is not acetylated in other parts of the pituitary and the brain.[79] β-Endorphin, which normally has a free N-terminus necessary to exhibit its morphomimetic character, becomes acetylated only in *pars intermedia*.[25,26,80] The N^{α}-acetyltransferase responsible for the N-terminal acetylation of these two polypeptides is present only in secretory granules in the intermediate pituitary of rat and ox.[70] N^{α}-acetylation has been shown to be linked to α-MSH release from *pars intermedia* of the amphibian pituitary gland.[81] It may well be that this acetylation of products which only come into existence after processing of a precursor protein is an exception, since for most proteins up to now acetylation has been shown to be a post-initiational process.

On the other hand, a shortage of acetyl coenzyme A may be responsible for incomplete acetylation. Jörnvall et al. showed that two isozymes of yeast alcohol dehydrogenase are synthesized with a consistent variation in acetylation under physiological conditions. Isozyme I is almost completely acetylated; isozyme II, which may be nonacetylated up to 40%, is the predominant form during aeration, when acetyl coenzyme A might be rapidly removed during efficient cell respiration. It has also been suggested that the availability of the coenzyme is a major factor in partial acetylation of human fetal hemoglobin, which has to compete with carbonic anhydrases B and C (both are N^{α}-acetylated) for the available acetyl groups in reticulocytes, where the main source of such groups, the mitochondrion, begins to disappear.[82] However, this case is more complicated because of an enhanced activity of the responsible acetyltransferase in erythrocytes as compared with reticulocytes.[83] Moreover, there is evidence that in vitro human fetal hemoglobin can be acetylated nonenzymatically.[84] This acetylation can be increased by sodium butyrate.[85] The latter compound seems to inhibit the turnover of preformed HbF_f. It is suggested that this is caused by inhibition of deacetylating enzymes. This action of sodium butyrate has been proven for histone N^{ϵ}-acetylation.[86]

The presence of deacetylases might be a third factor. Since there is evidence that N^{α}-acetylated proteins turn over at the same rate as nonacetylated ones,[7] it is possible that there exist such enzymes, which would make the protein accessible for aminopeptidases. However, up to now no enzyme removing N^{α}-acetyl groups from proteins has been found. On the other hand, the existence of enzymes deacylating N^{α}-acylamino acids has been established fairly well.[87-92] Much less is known about enzymes deacylating peptides. There are reports of N^{α}-acylpeptide hydrolases in liver[93] and erythrocytes.[94] These enzymes catalyze the hydrolysis of N^{α}-acylamino acids from the N-terminus of small peptides only. Remarkably, the human erythrocyte enzyme only releases acetylalanine, while the bovine liver enzyme also hydrolyzes acetylmethionine, -glycine, -serine, and -phenylalanine. Therefore, it has been suggested that the removal of acetyl groups from proteins may be affected by proteases which release an aminoterminally acetylated peptide, followed by an acylpeptide hydrolase which catalyzes the release of the *N*-acetylamino acid from the peptide and a deacetylase which hydrolyzes the acetylamino acid to yield acetate and the terminal amino acid.[94] Such enzymes are presumably involved in the catabolism of acetylated proteins. At the moment, there is no conclusive evidence that N^{α}-acetylated proteins are enzymatically deacetylated. Therefore, it is not very likely that deacetylation plays a role in the actual acetylation status of proteins, in general. Yet, in specific cases other factors may be involved. An intriguing example has been described for *E. coli*. This bacterium has a ribosomal protein which is found in both the N^{α}-acetylated (L7) and nonacetylated (L12) form.[95,96] Four copies of L7/L12 are present on the ribosome.[97] Ramagopal and Subramanian provided evidence that during early growth L12 is the major form present, while during the stationary phase L7 is

Table 3
NUMBER OF PROTEINS (UNIQUE GENE PRODUCTS) WHICH ARE N^{α}-ACETYLATED AND THE GROUPS TO WHICH THEY BELONG

Structural Proteins		48	40.7%
Actins	6		
Tropomyosin	2		
Intermediary filament proteins	2		
Keratins	8		
Virus coat proteins	9		
Crystallins	3		
Ribosomal proteins	4		
Histones	10		
Myelin proteins	2		
Others	2		
Enzymes		23	19.5%
Oxidoreductases	10		
Transferases	5		
Hydrolases	4		
Lyases	3		
Isomerases	1		
Transfer proteins		16	13.6%
Cytochrome c	4		
Hemoglobins	12		
Ca- and metal-binding proteins		16	13.6%
Parvalbumins	4		
Troponin C	3		
Myosin L-4 chain	1		
Calmodulin	1		
Intestinal Ca-binding protein	2		
S100b	1		
Metallothioneins	3		
Ferritin	1		
Hormones		3	2.5%
α-MSH	1		
β-Endorphin	2		
Miscellaneous proteins		12	10.1%
	12		

the predominant form.[98] This shift apparently requires production of new ribosomes, as it does not depend on modification of preassembled ribosomes.[99] In *E. coli* at least two, but a maximum of five, subspecies of the large subunits exist with regard to the L7/L12 content.[97] The observed variance in the L7/L12 ratio might reflect continuous changes in the selective abundance of these subspecies in vivo during the growth cycle. Moreover, it has been calculated that the level of the responsible N^{α}-acetyltransferase in *E. coli* remains constant during the growth cycle.[100] The possibility of a depletion of the available acetyl donor due to a high metabolism during growth should be investigated.

In conclusion, when N^{α}-acetyltransferase and acetyl coenzyme A are present, the most important factor in N^{α}-acetylation is the primary structure of the N-terminal region of the polypeptide chain.[16]

D. Which Proteins are N^{α}-Acetylated?

Until 1982, 361 proteins were described to be N-terminally acetylated, many of which are species variations of the same protein. When only unique genes are taken into account (using stringent selection rules) 118 proteins are left. Table 3 lists the variety of classes to

Table 4
ORIGIN OF N^{α}-ACETYLATED PROTEINS AS COMPARED WITH ALL SEQUENCED PROTEINS. FOR THE COMPARISON ONLY UNIQUE GENE PRODUCTS HAVE BEEN USED. THE NUMBER OF ENTRIES IS GIVEN BETWEEN BRACKETS

	Acetylated proteins (%) (entries)	All sequenced proteins (%)
Eukaryotes	86 (101)	77
Prokaryotes	4 (5)	18
Viruses	10 (12)	1
Bacteriophages	0 (0)	4

which they belong. About 40% are structural proteins. From the cumulative index of Supplement 3 of Dayhoff (1978)[1] we estimate that about 20% of all unique sequences are accounted for by structural proteins. Thus, N^{α}-acetylated polypeptides appear to be more often structural proteins than expected statistically. A predominance of structural proteins might be expected, if the function of N^{α}-acetylation, indeed, were protection against aminopeptidases. However, since there are many acetylated proteins with different size, origin, and function, this general property does not seem to be a limiting factor for acetylation.[16]

Only a few proteins of noneukaryotic origin are N^{α}-acetylated (Table 4). There are no examples of acetylated mitochondrial proteins, coded for by the mitochondrial genome,[101,102] nor acetylated polypeptides from bacteriophages. There are only five prokaryotic proteins N^{α}-acetylated, four of which are ribosome-associated. For viruses, 12 proteins have been described to be acetylated. When these data are compared with the distribution of these groups, over all unique gene products comprised in Dayhoff,[1] it is obvious that predominantly eukaryotic and proteins of eukaryotic viruses are N^{α}-acetylated. At the moment they comprise 96% of all known acetylated proteins. Data for viruses have to be considered with care, since many sequences have become available for this group since 1978. They might be underrepresented in the list of sequenced proteins of Dayhoff.[1]

Jörnvall et al. remarked that there are isozymes of bovine heart malate dehydrogenase and aldehyde dehydrogenase, in which the mitochondrial form is free, while the cytoplasmic form is N^{α}-acetylated.[30] They suggest that this reinforces an apparent link between nonacetylation and an environment with efficient aerobic respiration. There is another enzyme with isozymic forms for which this phenomenon has also been established. Whereas mitochondrial aspartate aminotransferase of pig and chicken heart is nonacetylated,[103-107] the cytoplasmic form is N^{α}-acetylated in chicken,[108,109] but nonacetylated in pig.[110] Virtually all Cu/Zn-superoxide dismutases are confined to eukaryotes,[112] whereas the Mn- and Fe-superoxide dismutases are widely distributed in eukaryotes, prokaryotes, and mitochondria. The latter are all related to each other, but not to the Cu/Zn family of superoxide dismutases. Harris et al.[111] interpret this similarity and the distribution in the light of the endosymbiontic hypothesis, which suggests that mitochondria of eukaryotes have evolved from bacteria which existed as intracellular symbionts in primitive eukaryotes.[112,113] This theory holds that when the atmosphere became more aerobic, these symbionts were acquired as a means of confronting selective pressures that require oxidative pathways.[114] Probably, the Cu/Zn type of enzyme evolved after the prokaryote-eukaryote divergence. In this respect it is remarkable that almost all Cu/Zn dismutases are N^{α}-acetylated, while the Mn and Fe dismutases are not.[1,111]

In conclusion, N^{α}-acetylation is a phenomenon predominantly confined to eukaryotes.

Table 5
N^{α}-TERMINAL AMINO ACID OF $N\alpha$-ACETYLATED PROTEINS (UNIQUE GENE PRODUCTS ONLY)

N terminus	Unique gene products	Percentage
Ac-Ser	41	34.8
Ac-Ala	38	32.2
Ac-Met	21	17.8
Ac-Gly	4	3.4
Ac-Asp	4	3.4
Ac-Glu	3	2.5
Ac-Tyr	2	1.7
Ac-Thr	2	1.7
Ac-Val	2	1.7
Ac-Asn	1	0.8
	n = 118	100.0

E. The N-Terminal Residue of N^{α}-Acetylated Proteins

When the 361 N^{α}-acetylated proteins, described up to 1982, are selected according to the stringent rules, the remaining 118 unique gene products (Table 5) mainly have Ala, Ser, and Met at the N-terminus (84.8%). Ala and Ser together make up 67.0% of the total. This is somewhat less than the 75% reported by Jörnvall.[16] However, it is obvious that methionine is found very often as N-terminal residue in acetylated proteins. While Jörnvall mentioned only four other residues to occur at the N-terminus of acetylated proteins (i.e., Asp, Gly, Thr, Val), we encountered in the literature the description of other N^{α}-acetylated N-termini such as Asn, Glu, and Tyr. Although there is a clear preference for Ser, Ala, and Met, there are still about 15% with other acetylated N-termini. This means qualitatively that at the aminoterminus of acetylated proteins half of the 20 amino acids can be found. The three amino acids Ala, Met, and Ser are all neutral; Ala and Ser are very similar in size, while Met is considerably larger. It is not known if this different bulkiness can be accommodated for by the active site of just one N^{α}-acetyltransferase. Remarkably, the larger hydroxyl amino acid Thr and the smaller aliphatic residue Gly, as compared to Ser and Ala, respectively, are not often present at the N-terminus of acetylated proteins.

The importance of the N-terminal residue is corroborated by the fact that the presence of a D-Ser at the N-terminus of an ACTH-fragment does not lead to N^{α}-acetylation in vitro.[57] This importance can further be demonstrated with several proteins which are found to be either nonacetylated or N^{α}-acetylated, depending on the nature of the aminoterminal residue. A convincing example has been found for the human mutant hemoglobin Raleigh. In this protein the β_1Val has been replaced by Ac-Ala.[23] The other examples which follow are more complicated due to differences other than the N-terminal residue. α-Globin normally has an unblocked Val or Met at position 1.[1] It is N^{α}-acetylated in fish and amphibians. In amphibians the N-terminal amino acid is Ala[115-117] or Ser,[118] in fish Ser.[1,119-121] Normally, mammalian β-globin has Val or Met at the N-terminus with a free amino group.[1] In the Felidae family different β-globins exist, some of which are blocked at the N-terminus.[122] In domestic cat the amino acid at position 1 has been shown to be Gly in case of the unblocked β-chain, and Ac-Ser in the case of the blocked chain.[17,113] β-Globins of some reptiles have Ac-Ala at the N-terminus.[124] Tobacco mosaic virus has a coat protein with either Ac-Ala or Ac-Ser at the N-terminus. However, one strain variant is known with unblocked Pro.[1] In cytochrome

Table 6
NUMBER OF PROTEINS, ACETYLATED AND NONACETYLATED, BELONGING TO EITHER THE EUKARYOTES OR THE PROKARYOTES

	Eukaryotes (viruses, mitochondria)	Prokaryotes (bacteriophages)
H-Ala, H-Met, H-Ser	136	114
Ac-Ala, Ac-Met, Ac-Ser	133	3

c, mammals have Ac-Gly-Asp and plants Ac-Ala-Ser at their N-terminus.[1] All other eukaryotic cytochromes c are nonacetylated. Insects have N-terminal Gly-Val, lower organisms Pro-Ala, Thr-Gln, and Gly-Phe.

Since there are many nonacetylated proteins which have the same N-terminal amino acids as N^{α}-acetylated proteins, other factors must be important for N^{α}-acetylation.

F. The N-Terminal Region of N^{α}-Acetylated Proteins

From the preceding sections it may be concluded that catalysis of N^{α}-acetylation in eukaryotes and prokaryotes is not achieved by acetyltransferases which have identical substrate specificity. However, N^{α}-acetyltransferases originating from different species or tissues recognize each other's substrates. Assuming that this is the general rule, we wanted to determine to what extent the primary structure of the N-terminal region is a decisive criterion for the suitability of a protein to be a substrate for N^{α}-acetylation. We, therefore, examined the first ten residues of proteins.

To this end we made use of our compilation of N^{α}-acetylated proteins (361 entries), taking into account only those which have N-terminal Ala, Ser, or Met (261 entries). These three were chosen, because they are the most frequently occurring N-terminal residues of acetylated proteins. For the nonacetylated proteins with the same N-terminal amino acids use was made of Dayhoff's protein sequence data set (312 entries). Both protein data sets were then screened with the less stringent selection rules. This left 136 entries in the case of the acetylated proteins and 250 of the nonacetylated ones.

The residual data sets were split up into entries from prokaryotic (including bacteriophageal) and eukaryotic (including viral and mitochondrial) origin. Since only a limited number of proteins is coded for by the mitochondrial genome,[101,102] of which none is included in our data sets, we placed all other mitochondrial proteins, encoded in the nucleus of the eukaryote, in the eukaryotic section. An investigation of the structural aspects of acetylated proteins from prokaryotic origin is not feasible, since only three are left after the selection. On the other hand, a sufficient number of sequences is known for eukaryotic proteins (compare Table 6).

Both the acetylated and nonacetylated proteins from eukaryotic origin were ordered alphabetically and compared. For each of the three amino acids Ala, Ser, and Met the sequences of both categories are given in the one-letter code up to the residue where a difference between them exists (Table 7). For the protein sequences now known, apparently, the decisive information for N^{α}-acetylation is enclosed within the first four amino acids for N-terminal Ala, within three for Met, and five for Ser. In the case of Ac-Met, there is a conspicuous preference for an acidic residue at position 2. In this respect it is remarkable that the methionine aminopeptidase normally responsible for the removal of the initiator Met does not act if position 2 is occupied by an acidic residue.[45] Unfortunately, these data do not yet allow the unambiguous prediction of the presence of acetyl groups in amino acid sequences derived from DNA-sequencing.

When we calculate the percentage of occurrence of amino acid residues for positions 2

Table 7
N-TERMINAL REGION OF ACETYLATED (AC-) AND NONACETYLATED (H-) PROTEINS, STARTING WITH ALA, MET, AND SER. RESIDUES ARE INDICATED WITH ONE-LETTER CODE[a]

Ac-	H-	Ac-	H-	Ac-	H-	Ac-	H-	Ac-	H-
AAQ	AAK	AKD	AKE		MA	SAN	SAG	SSK	SSSQD
AASI	AAN		AKG	MDA	MDM	SAQ	SAK	SSQ	SST
	AAP	ALK	ALT	MDD		SCY		SSSQK	
	AASG		ALV	MDG		SDA	SDEA	STA	STSR
	AAY		ALY	MDI		SDEE	SDH	STD	
ACC	ACD	AMT	AMS	MDK		SDQA	SDN	STST	
ACN			AN	MDL			SDP		SV
ACR			AP	MDP			SDQS	SYN	SYC
ADE	ADD	AQK	AQI	MDV		SEAP	SEAE	SYS	SYD
ADF	ADG	AQP		MEA	MEC	SELE	SEF		SYE
ADK	ADI		AR	MEE		SETAP	SELT		
ADQ	ADL	ASF	ASA	MEI			SES		
ADW	ADR	ASN	ASD		MH		SETAA		
	ADS	ASP	ASY		MI	SFA	SFD		
	ADT	ASQ		MKR	MKL	SFS			
AED	AEI	ATF	ATD		ML	SFT			
AEVA	AES	ATK	ATT	MN		SGR	SHG		
	AET	ATL	ATY		MQ	SGV			
	AEVL	ATP			MR	SH			
	AEVQ	AVA	AVE		MT	SI			
AFA	AFE		AVK			SKI	SKA		
AFG	AFKV		AVL			SKP	SKQ		
AFKG	AFP		AVQ			SL			
AGW	AGC		AVS			SN			
	AGQ		AVT			SRP	SRE		
AHL	AHR		AW						
	AI	AYN	AYD						
		AYS	AYR						

Note: The sequence is shown up to the residue where a difference between both classes is apparent; thus, the N-terminal sequence Ala-Ala-Gln (AAQ) has been found to be acetylated, whereas Ala-Ala-Lys (AAK) is not acetylated. AAS is found both acetylated and nonacetylated as N-terminal sequence in different proteins, but AASI has been found acetylated and AASG nonacetylated. Vertical bars indicate groups of sequences where the first two residues are identical. The groups are arranged alphabetically according to the N-terminal sequence in the one-letter notation. Frequently, the number of entries in acetylated and nonacetylated proteins within a group differ remarkably. In some cases there is only one representative in either one of these classes.

[a] Explanation of the one-letter code:[1] A = Ala, C = Cys, D = Asp, E = Glu, F = Phe, G = Gly, H = His, I = Ile, K = Lys, L = Leu, M = Met, N = Asn, P = Pro, Q = Gln, R = Arg, S = Ser, T = Thr, V = Val, W = Trp, Y = Tyr.

to 5 and 6 to 10 for both the acetylated and nonacetylated proteins and compare the results with the average percentage of these residues in proteins, in general (Dayhoff, 1978),[1] there seems to be a preference for Asp and Ile in residues 2 to 5 of acetylated proteins, while Gly, Gln, Arg, and Val are underrepresented (Table 8). In the nonacetylated proteins, Asp and Arg are overrepresented, while Ala, Gly, and Asn are underrepresented. Other extremely variable residues are not seen. We do not find the predominance of Ile, Leu, and Val as reported by Jörnvall,[16] and do not perceive a consistent pattern in the differences between acetylated and nonacetylated proteins. Only for Arg is the predominance in nonacetylated proteins balanced by an underrepresentation in acetylated proteins. In residues 6 to 10 of

Table 8
AVERAGE PERCENTAGE OF RESIDUES AT POSITIONS 2 TO 5 AND 6 TO 10 OF ACETYLATED AND NONACETYLATED PROTEINS, COMPARED WITH THE AVERAGE PERCENTAGE OF RESIDUES IN PROTEINS, IN GENERAL (DAYHOFF, 1978[1]). AMINO ACID RESIDUES ARE INDICATED WITH THE ONE-LETTER CODE

	H-		Ac-		
	2—5	6—10	2—5	6—10	Proteins, in general
A	5.9	5.6	9.4	12.6	8.6
C	2.6	3.2	2.6	2.6	2.9
D	11.7	6.4	9.4	7.3	5.5
E	6.8	8.2	6.9	5.5	6.0
F	2.3	5.7	4.9	3.6	3.6
G	4.2	7.7	5.6	7.7	8.4
H	2.0	2.6	1.3	2.6	2.0
I	3.4	1.5	7.1	4.2	4.5
K	5.2	6.0	8.5	9.4	6.6
L	6.7	8.2	6.6	4.7	7.4
M	2.0	1.0	0.6	0.8	1.7
N	2.6	1.7	3.6	4.0	4.3
P	5.3	7.9	5.6	8.1	5.2
Q	3.7	5.3	2.2	5.7	3.9
R	6.5	4.3	2.9	1.8	4.9
S	9.4	10.1	9.6	6.4	7.0
T	8.3	6.8	7.7	3.2	6.1
V	5.9	5.0	2.5	5.9	6.6
W	0.5	0.8	1.1	2.2	1.3
Y	5.0	2.0	1.9	1.7	3.4

both acetylated and nonacetylated proteins there are also apparent differences which are somewhat lower than for residues 2 to 5. Thus, differences tend to level off on going further along the chain. However, variations in residues 2 to 5 of acetylated and nonacetylated proteins are not consistent for the sequences hitherto known.

When we compare broad categories of amino acid residues for these same regions (Table 9), there seems to be a slight predominance of hydroxyl amino acids and acidic residues at positions 2 to 5 of acetylated sequences compared with proteins, in general. However, the same phenomenon is apparent in nonacetylated proteins. Small aliphatic amino acids are underrepresented in nonacetylated polypeptides. We do not believe that it is possible to carry out statistical quantitation, because our data sets are still rather small and contain overemphasis of related gene products.

In conclusion, there is no clear difference in steric and charge properties of the amino acid residues of positions 2 to 5 and 6 to 10 of acetylated and nonacetylated proteins starting with Ala, Ser, and Met. However, proteins starting with Ac-Met often have an acidic residue at position 2. Therefore, there seems to exist a difference between the structural requirements for acetylation of proteins starting either with Ala and Ser or Met.

Table 9
AVERAGE PERCENTAGE OF GROUPS OF RESIDUES AT POSITIONS 2 TO 5 AND 6 TO 10 OF ACETYLATED AND NONACETYLATED PROTEINS, COMPARED WITH THE AVERAGE PERCENTAGE OF THESE GROUPS IN PROTEINS IN GENERAL (DAYHOFF, 1978[1]). AMINO ACID RESIDUES ARE INDICATED WITH THE ONE-LETTER CODE

		H-		Ac-		
		2—5	6—10	2—5	6—10	Proteins, in general
Small aliphatic	A + G	10.1	14.0	15.0	20.3	16.9
Hydroxyl	S + T	17.7	16.9	17.3	9.6	13.1
Acidic	D + E	18.5	14.6	16.3	12.8	11.6
Acidic + acid amide	D + E + N + Q	24.8	21.6	22.1	22.5	19.8
Basic	H + K + R	13.7	12.9	12.7	13.8	13.5
Hydrophobic	I + L + M + V	18.0	15.7	16.8	15.6	20.2
Aromatic	F + W + Y	7.8	8.5	7.9	7.5	8.3

G. The Local Hydrophobicity of the N-Terminal Region of N^{α}-Acetylated Proteins

Since no obvious steric and charge properties account for the determination of the suitability of the N-terminal region of proteins for N^{α}-acetylation, we decided to investigate the local hydrophobicity of the first five amino acids of acetylated proteins, as the information for acetylation seems to be located in this region (Table 7). For the calculations we used the same data sets of acetylated and nonacetylated proteins as in the preceding section. The hydrophobicity of the three N-terminal residues involved differs. Met (+1.9) and Ala (+1.8) are comparable, while Ser (−0.8) is much more hydrophilic. In our calculations, we averaged the hydrophobicity over positions 1-2, 1-3, 1-4, and 1-5 for each protein. We then determined the average for all proteins. The results, expressed per residue, are summarized in Figure 2.

The most striking results are found for Met. While the N-terminal regions of proteins starting with H-Met show a relatively broad hydrophobicity range, the region of those with Ac-Met contains a moderate hydrophilic character for the first two positions ($\simeq -1.0$). Since Met itself has a value of +1.9, this means that the second residue is always very hydrophilic. There are no obvious differences on going further along the sequence. Since there are many proteins also having a hydrophilic residue at position 2 without being acetylated, other factors must be important, too, as was already deduced from Table 7. Average values for the first 5 positions tend to be somewhat more hydrophilic for acetylated proteins as compared with nonacetylated proteins. However, the values for span length 5 are already very close to each other.

When Ala is in the N-terminal position, we find that the hydrophobicity range between the highest and lowest value is near 4 for acetylated proteins for span lengths up to 5, while being somewhat larger (4 to 5) for nonacetylated proteins. The hydrophobicity per amino acid residue for acetylated proteins seems to be between the values for Phe and Pro. Extremely hydrophilic or hydrophobic N-terminal regions of proteins starting with Ala are not suitable for enzymatic N^{α}-acetylation. The same holds true for Ser. Values range between the same boundaries for acetylated proteins.

In conclusion, the hydrophobicity of the N-terminal region seems to play a role in N^{α}-acetylation. For Ala and Ser both very hydrophilic and hydrophobic N-terminal regions are unfavorable, while for Met position 2 must be very hydrophilic. Thus, again a difference between Ala/Ser, on the one hand, and Met, on the other, has been established.

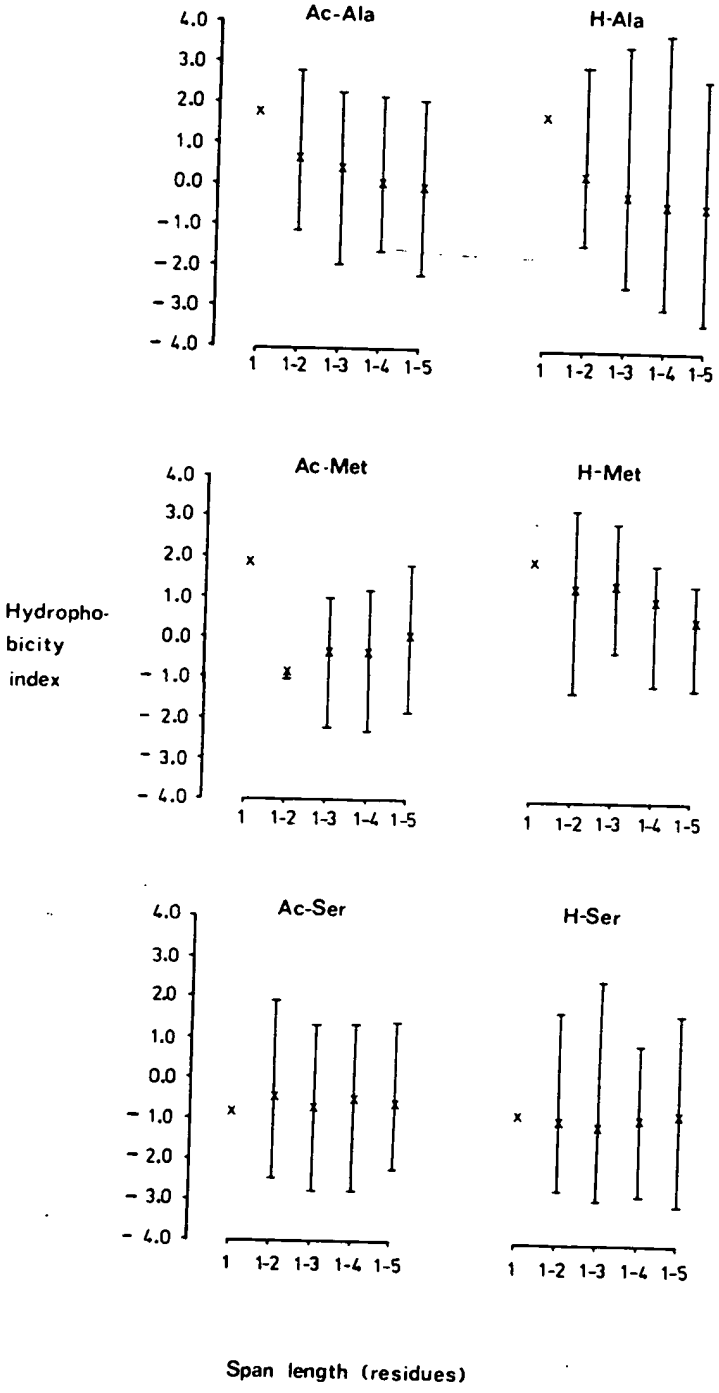

FIGURE 2. Hydrophobicity of the N-terminal region of proteins starting with Ac-Ala (n = 50), H-Ala (n = 87), Ac-Met (n = 27), H-Met (n = 19), Ac-Ser (n = 55), and H-Ser (n = 29). Results are shown per residue for span lengths of 2 up to 5. Average hydrophobicity for all proteins starting with a particular acetylated or nonacetylated residue is indicated with x. The highest and lowest values for any protein found are indicated with −.

H. The Two- and Three-Dimensional Structural Characteristics of N^{α}-Acetylated Proteins

For several of the known N^{α}-acetylated proteins information about the two- and three-dimensional structure is available. However, since N^{α}-acetylation mostly takes place with the nascent chain on the ribosome, when the chain starts to protrude at a length of 25 to 50 residues (mostly after removal of the initiator methionine), it is not clear to what extent, if any, the structure of the completed chain is of relevance for the N^{α}-acetylation process itself. In the completed chain the N-terminus is mostly present on the surface of the structure. The N^{α}-acetyl group is found at the beginning of an α-helix in dogfish lactate dehydrogenase M_4 isozyme[125] and at the beginning of a β-strand in lobster glyceraldehyde-3-phosphate dehydrogenase.[126] For the following proteins the N-terminal amino acid is separated by one or more residues from secondary structural characteristics: carp parvalbumin,[127] human glutathione reductase,[128] horse methemoglobin,[129] tuna ferricytochrome c,[130] human carbonic anhydrases B and C,[131,132] rabbit glycogen phosphorylase,[133] horse phosphoglycerate kinase,[134] horse liver alcohol dehydrogenase EE isozyme,[135] bovine Cu/Zn-superoxide dismutase,[136] and chicken muscle triosephosphate isomerase.[137] In the case of parvalbumin, the methyl carbon of the acetyl group is tucked back into the interior of the protein.

However, a completed three-dimensional structure does not have to be an impediment for efficient N^{α}-acetylation. Feline B-globin chains, synthesized in a rabbit reticulocyte lysate with prevention of acetylation[50] and isolated in normal tetrameric hemoglobin after addition of carrier protein, can be acetylated by incubation in the rabbit reticulocyte cell-free system. This has also been established for δ- and ε-actin, which are found in vitro and in vivo and are most likely nonacetylated precursors of cytoplasmic β- and γ-actin.[67,138] In vitro δ- and ε-actin can be acetylated very easily by incubation in cell-free systems in the presence of acetyl coenzyme A.

V. EXCEPTIONAL CASES OF N^{α}-ACETYLATION

As mentioned before, the products of proopiomelanocortin are acetylated post-translationally. The acetylation of α-MSH appears to be a very complicated process. α-MSH normally contains only one N^{α}-acetyl group. In 1979 Rudman et al. showed that in the pituitary of ox, rat, guinea pig, and rabbit one third of the melanotropic activity is represented by a structural variant of α-MSH with *N,O*-diacetylserine as its N-terminal residue.[139] When the pituitary gland is incubated in vitro, more than 90% of the melanotropic activity released in the medium is associated with the diacetylated compound. Physiological secretion of α-MSH may, therefore, be related to acetylation of the hydroxyl group of serine at position 1. In contrast to this assumption is the finding of Martens et al.[81] showing that in amphibian pituitary *pars intermedia* release of α-MSH is linked to N^{α}-acetylation. However, the latter authors did not verify the possibility that the compound, which was released, might be diacetylated. The separation of the mono- and diacetylated compound is very difficult.[139] The diacetylated compound is also present in porcine pituitary in equal amounts as in the other species.[140] Biological activity (melanotropic and lipolytic) is identical to that of α-MSH, so that the extra acetyl group on the hydroxyl oxygen of the N-terminal serine does not seem to have any effect in this respect. For bovine and rat pituitary the existence of this new melanotropin has been confirmed.[141] Glembotski has provided evidence that in rat *pars intermedia* ACTH is cleaved in CLIP (corticotropin-like intermediate lobe peptide) and ACTH-(1-13)-tridecapeptide (Figure 3).[70] The latter is then amidated C-terminally, followed by N^{α}-acetylation. Thereafter, the product, α-MSH, is acetylated at the hydroxyl group of the N-terminal serine. It is not known how many enzymes are responsible for these two acetylation steps.[61]

Another special case is actin. The N-terminal sequences of all actins are acidic and most likely acetylated[18,142,143] (compare the Appendix). In this respect actin is a conspicuous

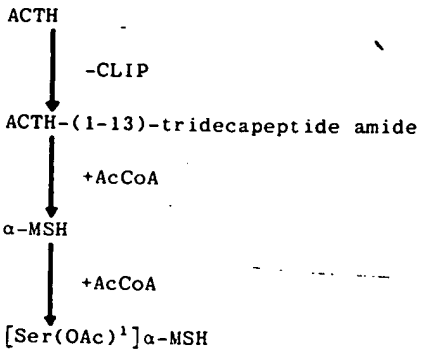

FIGURE 3. Processing of the N-terminus of ACTH in rat pituitary *pars intermedia*.

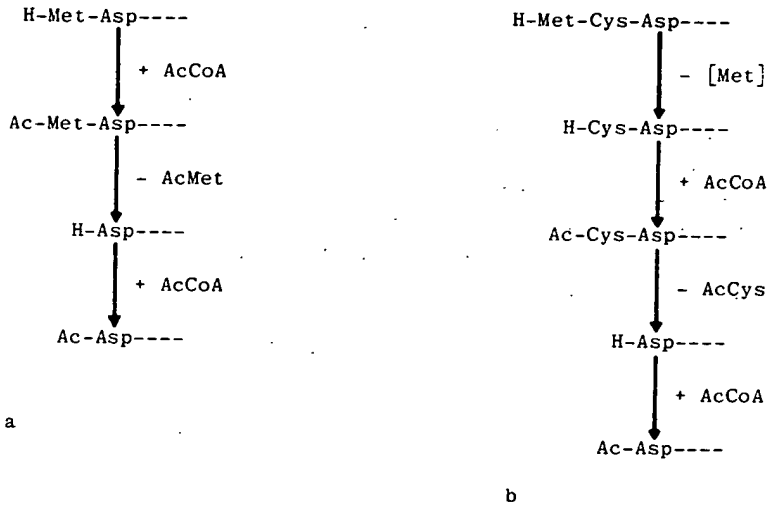

FIGURE 4. Processing of the N-terminus of actin from *Dictyostelium discoideum* (a) and *Drosophila melanogaster* (b) in vitro.

exception to the rules for N^{α}-acetylation as discussed in the preceding sections. As far as the processing of the N-terminal region of the actins is concerned, we have to discern between two groups of gene products. The first class comprises the cytoplasmic *Dictyostelium discoideum* and mammalian β-actins, where the initiator methionine immediately precedes the aspartic acid which is the N-terminal residue of the mature protein.[144-146] In the second class we find the mammalian striated muscle actins (cardiac and α-actin) and *Drosphila melanogaster* actin,[147-149] in which there are precursor proteins starting with Met-Cys-Asp. Since the mature proteins commence with the aspartic acid residue, the Met-Cys moiety has to be removed.

The N-terminal sequence of cytoplasmic actin from *Dictyostelium discoideum* is acidic and comprises the following sequence: Ac-Asp-Gly-Glu-Asp-....[18,68,69,142] Generally, in eukaryotic systems, the initiator methionine is removed if the second residue is not acidic.[45] It is, therefore, surprising that this type of cleavage does take place in the *Dictyostelium* actin. Rubenstein et al. showed that *Dictyostelium* actin prepared in a rabbit reticulocyte lysate in vitro contains Ac-Met at the N-terminus.[69] This product is rather stable. Post-translationally, Ac-Met is then removed (the acetyl group is necessary for this removal), followed by renewed acetylation.[68] It has been suggested[68] that this scheme (Figure 4A) is

also applicable for brain actin.[150] Interestingly, Met-Asp-Gly is a sequence which is often present among N^{α}-acetylated proteins with Met at the N-terminus (Table 7). The processing of *Drosophila melanogaster* actin in a reticulocyte lysate is somewhat different (Figure 4B).[151] At first, the initiator methionine is removed followed by N^{α}-acetylation of cysteine which possibly occurs while the nascent polypeptide is still attached to the ribosome. Further processing follows completion of the synthesis of the polypeptide. In a time-dependent fashion, Ac-Cys is removed, generating actin with an exposed aminoterminal aspartic acid which is subsequently acetylated to produce the mature form of actin.

Obviously, processing of actins in vitro has the novel feature of posttranslational removal of an N^{α}-acetylamino acid from the N-terminus of a completed peptide chain. Furthermore, the actins provide a second example of posttranslational acetylation besides the one already described for the products of proopiomelanocortin.

VI. CONCLUDING REMARKS

In summary, we have to admit that the mechanism of N^{α}-acetylation is still far from being fully understood. However, some features emerge from the data available:

- N^{α}-acetylation is a process virtually confined to eukaryotes.
- N^{α}-acetylation, in most cases, is a post-initiational process in which acetylation takes place when the nascent chain emerges from the ribosome.
- The nature of the N-terminal residue and its neighbors is crucial.
- N^{α}-acetyltransferases appear to have a narrow substrate specificity, which is almost identical for enzymes from different tissues and species.

In order to elucidate the precise function of N^{α}-acetylation further studies are of the utmost importance.

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